National Toxicology Program
Toxicity Report Series
Number 79

NTP Technical Report on the Reproductive Dose Range-Finding Toxicity Study of

Genistein

(CAS No. 446-72-0)

Administered in Feed to Sprague-Dawley Rats

October 2007

National Institutes of Health
Public Health Service
U.S. Department of Health and Human Services

FOREWORD

The National Toxicology Program (NTP) is an interagency program within the Public Health Service (PHS) of the Department of Health and Human Services (HHS) and is headquartered at the National Institute of Environmental Health Sciences of the National Institutes of Health (NIEHS/NIH). Three agencies contribute resources to the program: NIEHS/NIH, the National Institute for Occupational Safety and Health of the Centers for Disease Control and Prevention (NIOSH/CDC), and the National Center for Toxicological Research of the Food and Drug Administration (NCTR/FDA). Established in 1978, the NTP is charged with coordinating toxicological testing activities, strengthening the science base in toxicology, developing and validating improved testing methods, and providing information about potentially toxic substances to health regulatory and research agencies, scientific and medical communities, and the public.

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Administered in Feed to Sprague-Dawley Rats

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National Institutes of Health
Public Health Service
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The study on genistein was conducted at the FDA's National Center for Toxicological Research under an Interagency Agreement between the FDA and the NIEHS. The study was designed and monitored by a Toxicology Study Selection and Review Committee composed of representatives from the NCTR and other FDA product centers, NIEHS, and other *ad hoc* members from other government agencies and academia. The Interagency Agreement was designed to use the staff and facilities of the NCTR in the testing of FDA priority chemicals and to provide FDA scientists and regulatory policymakers information for hazard identification and risk assessment.

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PEER REVIEW

The draft report on the toxicity study of genistein was evaluated by the reviewers listed below. These reviewers serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, reviewers determine if the design and conditions of these NTP studies are appropriate and ensure that this Toxicity Study Report presents the experimental results and conclusions fully and clearly.

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CONTENTS

SUMMARY

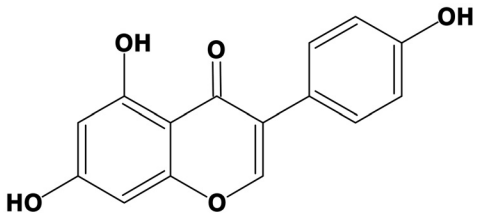
Background
Genistein is an isoflavone that occurs in soy products including soy-based infant formulas. Genistein is one of a class of chemicals known as "environmental estrogens" that can affect the hormone activities and possibly reproductive function of wildlife and humans through exposure. The NTP conducted a series of studies on three such chemicals to detect if exposure to such chemicals over the course of multiple generations could have any cumulative effect on animals' reproductive systems or development of cancers. This report describes a preliminary short-term study to assess the effects of genistein on rats and to determine appropriate doses to be used in the multiple-generation long-term studies.

Methods
We gave groups of 10 pregnant female rats genistein mixed in their feed at concentrations of 5, 25, 100, 250, 625, or 1,250 parts per million (ppm), continued the diet until they gave birth and through lactation, and then gave their pups the same diet until 50 days after birth. The animals were then examined for changes in their body and organ weights, reproductive system, immune system, or neuroanatomy.

Results
Both the mothers and the pups exposed to the highest concentration of genistein had lower body weights at the end of the studies. Female pups receiving 250 ppm or more developed hyperplasia of the mammary glands; male pups exposed to as little as 25 ppm developed hypertrophy of the mammary gland, and male pups receiving 250 ppm also developed hyperplasia of the mammary gland. There was an increase in vaginal metaplasia in female pups receiving 625 ppm or more.

Conclusion
Exposure of mother and pup rats to genistein resulted in precancerous lesions of the mammary gland in both sexes of pups and of the reproductive system in female pups. From this study, doses of 625 ppm were considered too high for long-term studies, so maximum doses of 500 ppm genistein in feed were selected for subsequent multigenerational studies.

ABSTRACT



GENISTEIN

CAS No. 446-72-0

Chemical Formula: $C_{15}H_{10}O_5$ Molecular Weight: 270.23

Synonym: 4′,5,7-Trihydroxyisoflavone

Genistein is a naturally occurring isoflavone that interacts with estrogen receptors and multiple other molecular targets. Human exposure to genistein is predominantly through consumption of soy products, including soy-based infant formula and dietary supplements. A series of short-term studies with genistein was conducted with two goals: 1) to obtain data necessary to establish dose levels for subsequent multigeneration reproductive and chronic toxicity studies and 2) to evaluate the effects of genistein on endpoints outside the reproductive tract. The data generated from these studies have been reported previously in the peer-reviewed literature or in technical reports (Appendix C). In addition, selected data from these studies were analyzed and discussed in the National Toxicology Program's Report of the Endocrine Disruptors Low-Dose Peer Review (NTP, 2001). The present report focuses on the reproductive and general toxicology endpoints evaluated. Data obtained in separate evaluations of behavioral, neuroanatomical, neurochemical, and immunological endpoints, as well as the assessment of serum genistein levels, are also discussed to put in better perspective the selection of doses for the multigenerational and chronic studies.

Genistein was administered in an irradiated soy- and alfalfa-free diet (Purina 5K96) at exposure concentrations of 0, 5, 25, 100, 250, 625, or 1,250 ppm to 10 vaginal plug-positive, female Sprague-Dawley rats starting on gestation day 7 and continuing throughout pregnancy. These dietary exposure concentrations resulted in ingested doses of approximately 0.3, 1.7, 6.4, 16, 38, and 72 mg genistein/kg body weight to dams in the 5, 25, 100, 250, 625, and 1,250 ppm groups, respectively. Dietary exposure of the dams continued through lactation, during which time

ingested doses were approximately 0.6, 3.5, 14, 37, 84, and 167 mg/kg per day. Pups from five litters, culled to eight per litter with an equal sex distribution on postnatal day (PND) 2, were maintained on the same dosed feed as their mothers after weaning until sacrifice at PND 50. Ingested doses were approximately 0.6, 3, 11, 29, 69, and 166 mg/kg per day for male pups and 0.6, 3, 12, 31, 73, and 166 mg/kg per day for female pups. Body weight and feed consumption of the treated dams prior to parturition showed decreasing trends with increasing dose, and both parameters were significantly less than those of the controls in the 1,250 ppm group. A significant exposure concentration-related effect on litter birth weight was observed, but no exposed group differed significantly from the control group in pairwise comparisons. Pups in the 1,250 ppm group had significantly decreased body weights relative to controls at the time of sacrifice (males, 9% decrease; females, 12% decrease). The most pronounced organ weight effects in the pups were decreased ventral prostate weight (absolute weight, 28% decrease; relative weight, 20% decrease) in males at 1,250 ppm and a trend toward higher pituitary gland to body weight ratios in both sexes. Histopathologic examination of female pups revealed ductal/alveolar hyperplasia of the mammary glands at exposure concentrations greater than 250 ppm. Ductal/alveolar hyperplasia and hypertrophy also occurred in males, with significant effects seen at exposure concentrations of 25 ppm or greater for hypertrophy and 250 ppm or greater for hyperplasia. Abnormal cellular maturation (mucocyte metaplasia) in the vagina was observed at 625 and 1,250 ppm, and abnormal ovarian antral follicles were observed at 1,250 ppm. In males, aberrant or delayed spermatogenesis in the seminiferous tubules relative to controls was observed at 1,250 ppm. Histologic evaluation indicated a deficit of sperm in the epididymis at 625 and 1,250 ppm relative to controls, although testicular spermatid head counts and epididymal spermatozoa counts did not show significant differences from controls at these exposure concentrations. Control females showed a high incidence of renal tubule mineralization, and the severity of this lesion was significantly increased at exposure concentrations of 250 ppm or greater. Males showed no renal tubule mineralization below 250 ppm, but incidence and severity increased with increasing exposure concentration at 250 ppm and greater.

The primary goal of the current study was to provide information for the selection of exposure concentrations to be used in subsequent multigenerational and chronic studies. These long-term studies were designed to address multiple aspects of the endocrine disruptor hypothesis, that is, the hypothesis that exposures of human and wildlife populations to endocrine-active compounds contribute to adverse reproductive tract effects and cancers of hormone-sensitive organs. In particular, the long-term consequences of low dose exposures that may produce subtle initial effects, the magnification of those effects across generations, and the reversibility of those effects were to be investigated. The goal was to select a high exposure concentration that would not induce overt toxicity in the dams or pups but would induce observable effects in the reproductive organs of the pups without severely impairing fertility in the F_1 generation. The 1,250 ppm exposure concentration was clearly ruled out for further testing based on the effects on body weights, histopathologic observations in males and females, and a reduction in the proportion

of mated dams producing litters. While the effects observed at 625 ppm would not be predicted to significantly impair reproduction, the observation of significant effects at 250 ppm (hyperplasia in the mammary gland of both sexes), together with the suggestion of subtle effects at this exposure concentration and less in the parallel immunotoxicity and neuroanatomical surveys, a high exposure concentration between 250 and 625 ppm was deemed appropriate for the purposes of the multigenerational reproductive toxicology study and the chronic study of genistein. A high exposure concentration for the multigenerational and chronic studies was thus set at 500 ppm. A low exposure concentration of 5 ppm, where no significant effects were observed in the reproductive dose range-finding, and an intermediate exposure concentration of 100 ppm were also selected.

OVERVIEW OF THE ENDOCRINE DISRUPTOR STUDIES

ENDOCRINE DISRUPTORS

Numerous chemicals have been implicated as potential modulators of the activity of endogenous hormones in wildlife and humans either through direct interaction with hormone receptors or by modifying hormone synthesis or metabolism (Colborn and Clement 1992; Colborn *et al.,* 1993). Because of the wide range of physiological processes that are influenced by endogenous hormones, such modulating chemicals could have a multiplicity of effects, including effects on reproduction, nervous system function and behavior, immune responses, bone, the cardiovascular system, and carcinogenesis. A large body of work on long-term reproductive organ toxicity, including carcinogenesis, in animals and humans exposed *in utero* or perinatally to the potent estrogen diethylstilbesterol (DES) has underlined the potential consequences of exposure to hormonally active agents at critical time periods in development (Herbst *et al.,* 1971; Gill *et al.,* 1977; Robboy *et al.,* 1977; McLachlan *et al.,* 1980; Newbold *et al. ,*1990; Santti *et al.,* 1994). These data led to the hypothesis that exposure to endocrine-disrupting chemicals during development has been and continues to be a contributory factor to human reproductive problems (e.g., reduced sperm counts, reproductive tract malformations), reproductive tract cancers, and other endocrine-influenced endpoints. Chemicals with the ability to modulate endocrine signaling pathways are widespread and occur naturally (e.g., phytoestrogens), as well as being produced synthetically for use in a variety of products, including pesticides used on crops and animals and in the production of polymers used in food packaging materials. While the association between exposure to pharmacological doses of DES at critical developmental time points and adverse effects in humans has been clearly defined, the association of lower levels of less potent hormonally active agents, or endocrine disruptors, and toxicity in humans has not been clearly documented (NRC, 1999).

Following a 1994 meeting sponsored by the National Institute of Environmental Health Sciences (NIEHS) entitled "Estrogens in the Environment III," NIEHS (1995) proposed to expand and develop mammalian animal models to determine if environmentally relevant doses of endocrine-disrupting chemicals and mixtures of these chemicals during exposure windows that included development could cause reproductive problems or influence the incidence of reproductive tract cancers. Investigation of the potential for magnification of subtle reproductive effects over multiple generations, the importance of exposure windows, and whether effects are reversible or are imprinted to carry over across generations were also deemed to be important. The utility of such a program was endorsed by the National Toxicology Program (NTP) Board of Scientific Counselors at their meeting on October 18, 1994. The

series of studies related to this initiative were conducted under Interagency Agreement between the NIEHS/NTP and the Food and Drug Administration/National Center for Toxicological Research (FDA/NCTR). Study protocols were generated and dose range-finding studies were initiated at NCTR in 1997.

Study Rationale and General Study Design

The overall goal of this series of studies was to evaluate the long-term consequences of doses of endocrine-active agents that produced subtle, short-term effects in exposed animals. The idea behind the studies was to evaluate aspects of the "endocrine disruptor hypothesis," which is the hypothesis that environmental exposure to endocrine-active chemicals is contributing to a variety of adverse effects in wildlife and humans (NRC, 1999). As originally conceived, the plan was to evaluate neurobiological, behavioral, immunological, reproductive, and chronic toxicities in the main studies. This plan was modified to assess all of these endpoints in short-term studies prior to the main studies that focused on reproductive and chronic toxicity. The compounds selected for multigenerational studies were three agents that vary in estrogenic potency: the soy isoflavone, genistein; the industrial intermediate, *p*-nonylphenol; and the potent and widely used synthetic estrogen, ethinyl estradiol.

A short-term dose range-finding study was conducted for each compound to assess general and reproductive toxicity, behavioral toxicity, neurotoxicity, and immunotoxicity. Pregnant females were given dosed feed (a soy- and alfalfa-free rodent diet) from gestation day 7 (GD 7) until the pups were weaned, and the pups were continued on the same diet as their dams until termination. Separate sets of animals were bred for the reproductive, behavioral, and immunological studies. One pup per sex per litter from the reproductive study was used for the neurotoxicity study. Data from the reproductive/general toxicity study were the primary data used for selection of exposure concentrations for the subsequent multigenerational reproductive toxicology and chronic studies, although data from the other studies were considered in choosing the range of exposure concentrations to be tested. All of these studies, as well as the subsequent multigenerational and chronic studies, utilized outbred CD (Sprague-Dawley) rats from the NCTR breeding colony. The Sprague-Dawley rat was selected because of its widespread use in reproductive toxicology studies, including those conducted by the NTP, its robust breeding performance, and its relatively low background incidences of testicular Leydig cell tumors and large granular lymphocyte leukemia relative to the F344 rat commonly used in NTP carcinogenesis studies. The relatively high background incidences of pituitary gland and female mammary gland tumors in Sprague-Dawley rats were recognized as a possible concern. The relatively poor breeding performance of the F344 rat would have presented a considerable challenge to the conduct of the studies described here, as it would for any evaluation of reproductive toxicity. Reproductive toxicity testing guidelines (for example, those of the EPA, FDA, and OECD) generally indicate that animals with low fecundity not be used. While the studies described in this report were underway, the use of the Sprague-Dawley rat, particularly the Charles River

CD (SD) rat, to screen for endocrine-active substances has been a subject of controversy due to the reported lower sensitivity of this strain to estrogenic agents for some endpoints relative to other strains, including the F344 (Parker and Tyl, 2003; Spearow, 2004). On the other hand, there is some evidence that this strain may be more sensitive to the estrogen receptor-mediated responses to tamoxifen than the F344 rat (Bailey and Nephew, 2002), and the response of various strains to estrogens appears to depend on the endpoint and the agent (Parker and Tyl, 2003; Spearow, 2004). This debate continues, and there is no consensus at this time as to what strain or combination of strains would be optimal for screening for endocrine-active agents. The Sprague-Dawley rat continues to be used widely in reproductive toxicity studies. As mentioned previously, the present studies utilized outbred female CD (Sprague-Dawley) rats from the NCTR breeding colony. This colony was established at NCTR in 1972 using Sprague-Dawley rats from the Charles River Laboratories. The NCTR colony at present is a distinct substrain of Sprague-Dawley rat and has been previously shown to differ substantially from the Charles River and other strains of SD rat in terms of body weight, which is lower than that reported for other substrains, and survival, which is longer than that reported for other substrains (Duffy *et al.,* 2001). The sensitivity of the NCTR CD rat to the potent estrogen ethinyl estradiol was evaluated as part of this series of studies and reported separately (NTP, 2008a,b).

It was intended that exposure concentrations that were within the range of human exposures and/or below previously reported no-observed-adverse-effect-levels be incorporated in the main studies. The experimental design was intended to determine if subtle effects would be magnified in subsequent generations and if observed effects were reversible. In standard reproductive toxicity studies conducted for regulatory purposes, high doses are chosen to produce some maternal toxicity, while the low dose is selected with the goal of not producing parental effects (CFSAN, 2000; OECD, 2004). The high dose for chronic studies is set as the maximum tolerated dose. In the present series of studies, the goal was to select a high dose, based on the results of the reproductive toxicity dose range-finding study, that did not produce significant maternal toxicity but did produce reproductive tract lesions in the offspring of a degree that would not severely affect reproductive capacity in the first generation. The questions addressed in the chronic studies were whether doses producing subtle modifications of the reproductive tract could produce chronic toxicity and whether any observed chronic toxicity was induced by early developmental exposure or rather required continuous long-term exposure.

The need to maintain consistent dietary composition was taken into account in the design of this series of studies. A soy- and alfalfa-free diet (PMI 5K96) with consistently low concentrations of the phytoestrogens genistein and daidzein was utilized in all studies. A preliminary study indicated that rats fed this diet had reproductive capacity equivalent to rats fed NIH-31 diet, the standard soy- and alfalfa-containing diet used at the test facility (NCTR), although feed consumption in both sexes and the body weights of males fed PMI 5K96 were significantly lower than in rats fed NIH-31.

Design of the Multigenerational Reproductive Toxicology and Chronic Studies Conducted Subsequent to the Dose Range-Finding Studies

As in the short-term studies, the multigenerational reproductive toxicology and chronic studies were conducted with the NCTR CD (Sprague-Dawley) rat and test compounds were administered in the soy- and alfalfa-free 5K96 diet. The design of the multigenerational reproductive toxicology and chronic studies is outlined in Figure 1. For the multigenerational reproductive toxicology studies, males and females of the original parental generation (F_0) were placed on the 5K96 diet at weaning, and dosed feed was administered starting on PND 42, approximately 1 month before breeding. The F_0 generation was maintained on dosed feed until termination at PND 140. For breeding, one male was cohabited with one female for 14 days or until a vaginal plug (*in situ* or in pan below cage) was detected. Subsequent generations (F_1 through F_4) were bred similarly. The F_1 and F_2 generations were exposed to the test compound administered in the diet continuously from conception through termination at PND 140; the F_3 generation was removed from exposure at weaning (PND 21) and continued on control feed until PND 140, while the F_4 generation received no dietary exposure to the test compound. The F_4 generation was bred to produce an unexposed F_5 generation. The F_5 litters were terminated at weaning following collection of basic litter information. Standard toxicological data and reproductive development and performance data were collected for all generations, and organ weights and histopathology data were collected for 25 randomly selected animals per sex per exposure concentration for each generation at necropsy.

Chronic toxicity, which is reported separately, was also examined for two test compounds (genistein and ethinyl estradiol). Three exposure windows were examined in the chronic studies (Figure 1): continuous exposure from conception through 2 years (designated F_1 continuous, or F_1C), exposure from conception through PND 140 followed by control diet to 2 years (designated F_1 truncated at PND 140, or F_1T140), and exposure from conception through weaning followed by control diet to 2 years (designated F_3 truncated at PND 21, or F_3T21). The F_3 designation for the F_3T21 exposure groups indicates that these animals were siblings of the F_3 animals from the multigenerational reproductive toxicology study. Because of the number of animals required for the chronic study of each test chemical, separate sets of animals were used for the multigenerational reproductive toxicology study and the F_1 generation chronic study. The assessment of chronic toxicity resulting from dietary exposure from conception through weaning was conducted with animals from the F_3 generation of the multigenerational reproductive toxicology study.

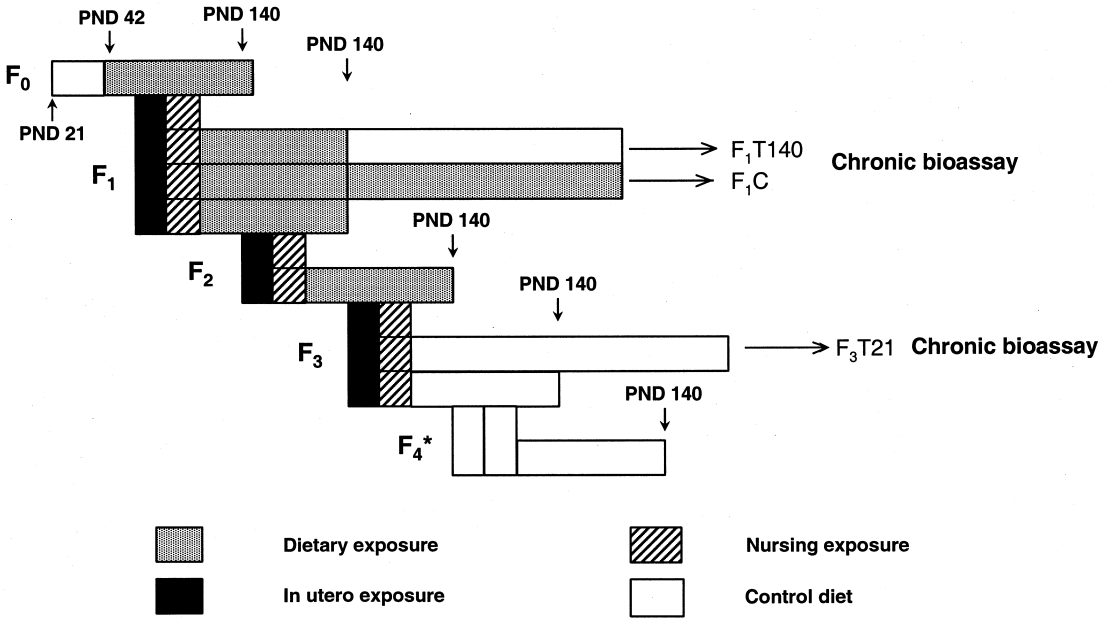
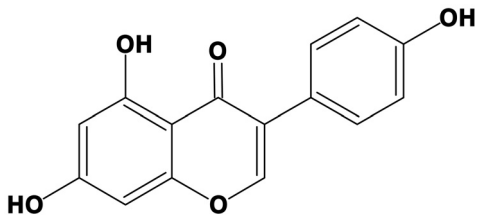


FIGURE 1
Dosing Schedule for the Multigenerational Reproductive Toxicology and Chronic Studies
* The F_4 generation was mated similarly to generations F_0 to F_3 to produce the F_5 generation

INTRODUCTION



GENISTEIN

CAS No. 446-72-0

Chemical Formula: $C_{15}H_{10}O_5$ Molecular Weight: 270.23

Synonym: 4′,5,7-Trihydroxyisoflavone

PHYSICAL PROPERTIES, PRODUCTION, USE, AND EXPOSURE

Genistein belongs to the class of chemicals designated isoflavones. It has a molecular weight of 270.23 and in pure form is a pale-yellow crystalline solid that is practically insoluble in water but freely soluble in methanol and ethanol (*Merck,* 1996). In nature, genistein is primarily found in legumes where it is produced by a branch of the phenylpropanoid pathway of secondary metabolism through the action of the enzyme isoflavone synthase on the flavanone intermediate naringenin (Dixon and Ferreira, 2002; Jackson and Rupasinghe, 2002). Products derived from soybeans are the primary source of human exposure to genistein. Genistein content of soybeans varies according to the cultivar and season, and processing of the soybean and soyfoods further affects both the genistein content and the form of genistein present (Gugger, 2002; Jackson and Rupasinghe, 2002). The aglycone form of genistein (shown above) is present primarily in fermented products such as miso and tempeh, whereas in whole soybean and nonfermented products such as tofu or soy drinks, genistein exists predominately as the glucoside conjugate (genistin) or acetyl or malonyl derivatives of genistin. Glucosides and glucoside derivatives are hydrolyzed to the aglycone genistein in the gut by gut bacteria or gut wall enzymes.

Intake patterns and isoflavone content of ingested products vary widely, but the Committee on Toxicity of Chemicals in Food Consumer Products and the Environment of the United Kingdom has recently estimated an approximate rank order of daily isoflavone exposure as follows: infant on soy formula (40 mg genistein/day), average Japanese

consumer (25 to 100 mg/day), vegetarian consumer (3 mg/day), and the average British consumer (1 mg/day) (CTCF, 2003). The typical ingestion by the average consumer in the United States is likely to be similar to that by a British consumer. Data on isoflavone intake from dietary supplements are sparse, but the CTCF estimated that manufacturers' recommended daily dosages would result in exposures of 29 to 88 mg isoflavones per day, or about 0.4 to 1.3 mg/kg per day for a 70 kg person. On a body weight basis, infants consuming soy formula are exposed to the highest doses, with mean doses estimated to be 6 to 9 mg/kg per day (Setchell *et al.,* 1997).

The consumption of diets with high levels of soy has been proposed to have multiple beneficial effects, including chemopreventive activities against various cancers and alleviation of some of the adverse consequences of menopause, although the epidemiological evidence for many of these beneficial effects is controversial (Adlercreutz, 2002; Messina *et al.,* 2006; Sacks *et al.,* 2006; Trock *et al.,* 2006; Williamson-Hughes *et al.,* 2006). Diets high in soy contain multiple agents that may contribute to these effects, and consumption of these diets is also associated with lower calorie and fat intake. Nonetheless, much research attention has focused on the isoflavones, and particularly genistein, as the active components contributing to (or responsible for) the beneficial effects of soy. This is due to the demonstrated interaction of soy isoflavones, particularly genistein, with estrogen receptors, effects on hormone synthesis and metabolism and sex hormone binding proteins, and genistein's ability to modulate multiple enzymes involved in growth regulation, including tyrosine kinases and topoisomerases. These activities have been extensively reviewed (see above references). Genistein has been demonstrated in numerous studies to act as an estrogen by stimulating uterine growth in immature or ovariectomized rodents and has been shown to induce a similar, though not identical, pattern of gene expression as ethinyl estradiol in the developing rat uterus (Naciff *et al.,* 2002) and in developing rat testes and epididymides (Naciff *et al.*, 2005). Recent studies, published after the present study was completed, have also indicated that genistein at concentrations above 1 µM can modulate the expression of androgen-regulated genes and peroxisome proliferator activated receptor-α- and -γ-regulated genes (Dang *et al.,* 2003; Mezei *et al.,* 2003; Takahashi *et al.,* 2004; Kim *et al.,* 2005), thus adding to the potential complexity of genistein-mediated effects. The association of diets containing soy with lower rates of many common Western health problems has led to the development of concentrated isoflavone-containing plant extracts for use as dietary supplements (Hodgson *et al.,* 1998; Nestel *et al.,* 1999; Kurzer, 2003). In addition, soy-based infant formulas have been available for decades, and infants consuming soy formula have been shown to have concentrations of circulating isoflavones as high as 5 to 10 µM (Setchell *et al.,* 1997).

Research assessing the potential adverse effects associated with isoflavone consumption is directed toward defining any potential risk from exposure to a range of doses of isoflavones during different life stages. Developmental stages are of particular concern because of the demonstrated adverse consequences of exposure to hormonally active agents, such as diethylstilbestrol, during development (Bern, 1992; Newbold, 1995; NIH, 1999), although potential adverse

stimulatory effects of genistein on reproductive and breast tissues of postmenopausal women also require particular attention (Petrakis *et al.,* 1996; Hargreaves *et al.,* 1999). With regard to potentially estrogenic effects of genistein in estrogen-responsive tissues in humans, several studies in female nonhuman primates have failed to demonstrate estrogenic effects of soy protein isolate or soy isoflavone mixtures that included genistein up to doses approximating 10 mg/kg per day genistein and resulted in serum levels of genistein equivalent to those achieved from high dose soy ingestion in women (Wood *et al.,* 2004, 2006a,b,c).

Adverse effects of soy-containing foods and soy components on reproductive processes of animals had been reported prior to the initiation of this study (East, 1955; Stob, 1983; Price and Fenwick, 1985), and some human studies had suggested that the consumption of soy products can have hormonal effects in women (Wilcox *et al.,* 1990; Cassidy *et al.,* 1994; Baird *et al.,* 1995; Cassidy and Bingham, 1995; Nagata *et al.*, 1997, 1998; Xu *et al.,* 1998; Duncan *et al.,* 1999). It has been suggested further, based on studies in ovariectomized rodents and nonhuman primates, that beneficial effects of soy and its component isoflavones on the cardiovascular system and bone occur at doses that do not adversely affect the reproductive tract (Anthony *et al.,* 1996; Ishimi *et al.,* 1999). In addition, inhibition of chemically induced mammary cancer in rats has been reported at doses that did not produce adverse effects on reproductive tissues (Murrill *et al.,* 1996; Fritz *et al.,* 1998). Given the potential range of effects of soy and its components and the magnitude of human exposure, it is important to conduct comprehensive toxicological evaluations of these agents to better understand potential adverse effects that could result from their use in products such as dietary supplements and soy infant formula.

GENETIC TOXICITY

While much of the focus on the potential induction or modulation of cancer development by genistein has been on its activity as a phytoestrogen, the potential for genotoxicity has also been evaluated. Genistein has been tested for mutagenicity in *Salmonella typhimurium* strains TA97, TA98, TA100, TA102, TA1535, and TA1538 with and without a rat liver S9 metabolic activation system, and the results were negative (Bartholomew and Ryan, 1980; Nagao *et al.,* 1981; McClain *et al.,* 2006). Misra *et al.* (2002) also reported a statistically significant but modest (less than twofold) positive mutagenic response in TA100 with S9 activation using an isoflavone mixture containing 40% to 50% genistein, 18% to 25% daidzein, and 1% to 4% glycitein.

Genistein has been shown to bind to DNA topoisomerase II to produce DNA strand breaks (Markovits *et al.,* 1989; Snyder and Gillies, 2002). DNA strand breaks, micronucleus formation, and mutations at the hypoxanthine phosphoribosyltransferase and thymidine kinase genes in mammalian cells *in vitro* have been reported with and without coincubation with a rat liver S9 metabolic activation system at concentrations as low as 3 to 5 µM (Yamashita *et al.,* 1990; Record *et al.,* 1995; Kulling and Metzler, 1997; Morris *et al.,* 1998; Kulling *et al.,* 1999; Boos and

Stopper, 2000; Snyder and Gillies, 2003; Di Virgilio *et al.,* 2004; McClain *et al.,* 2006). Of particular concern was the demonstration of genistein-induced translocations and deletions in the mixed-lineage leukemia (MLL) gene in hematopoietic mononuclear cells isolated from umbilical cord blood, because this gene is associated with acute myelogenous leukemia (AML) (Strick *et al.,* 2000). Epidemiological studies have been interpreted to suggest a possible association between consumption of diets high in DNA topoisomerase II inhibitors, although not necessarily genistein, with the development of MLL-positive AML (Ross *et al.,* 1996; Spector *et al.,* 2005). *In vivo* studies with genistein in mice and rats, however, have not demonstrated an increase in micronucleus frequency (Record *et al.,* 1995; Misra *et al.,* 2002; McClain *et al.,* 2006) nor an increase in mutations in the lac I or cII genes of several organs of transgenic Big Blue rats (Chen *et al.,* 2005; Manjanatha *et al.,* 2005). Misra *et al.* (2002) reported that an isoflavone mixture containing 40% to 50% genistein, 18% to 25% daidzein, and 1% to 4% glycitein did not enhance neoplasm formation in p53 knockout mice at a dietary concentration that delivered 50 to 60 mg genistein/kg body weight per day. A subsequent gavage study indicated that this isoflavone mixture did not induce neoplasms in any organ in p53 knockout mice at doses up to 2,500 mg/kg per day for 6 months (Johnson *et al.,* 2006). Morris *et al.* (2003) did report an increasing proportion of small intestine cells in S-phase and a decreasing proportion in G_0 over an exposure concentration range of 100 to 2,000 ppm dietary genistein (approximately 17 to 460 mg/kg per day) in C57BL6 mice, which they interpreted as consistent with *in vivo* inhibition of DNA topoisomerase II. A clinical study in 20 prostate-cancer patients who received 300 mg genistein (approximately 4 mg/kg per day) for 28 days followed by 600 mg genistein per day for 56 days was reported by Miltyk *et al.* (2003). These doses resulted in plasma levels of total genistein ranging from 4 to 27 µM and aglycone levels ranging from 0.02 to 0.32 µM. There was no evidence of increases in DNA strand breaks, micronucleus formation, or translocations in the MLL gene in peripheral lymphocytes. Thus, while the ability of genistein to induce chromosomal damage has been clearly demonstrated in *in vitro* systems, conditions under which such damage may be induced *in vivo* have not been demonstrated. In addition, genistein was reported to be negative in the *in vitro* Syrian hamster embryo cell transformation assay (Harvey *et al.,* 2005).

STUDY RATIONALE AND DESIGN

Given the potential range of effects of soy and its components and the magnitude of human intake, comprehensive toxicological evaluations of these agents are needed to better understand potential adverse effects that could result from their use. The purpose of the present study was to provide data for selection of the dietary levels of genistein to be used in subsequent multigenerational and chronic toxicity studies. Dose levels for the dose range-finding study were selected to cover both human isoflavone exposure levels and doses, on a body weight basis, that had been reported in previous studies to have effects on target organs. The doses selected (0, 5, 25, 100, 250, 625, and 1,250 ppm) were anticipated to give a range of exposures from less than 1 mg/kg per day to greater than 50 mg/kg per day. The actual ingested doses of genistein calculated from food consumption and body weight data at various stages of the experiment are shown in Table 1.

TABLE 1
Mean Genistein Intake for Dams and Pups Calculated from Feed Intake and Body Weight Data[a]

	0 ppm	5 ppm	25 ppm	100 ppm	250 ppm	625 ppm	1,250 ppm
Pregnant dams							
(GD 7 to parturition)	0	0.3 ± 0.0	1.7 ± 0.2	6.4 ± 1.0	15.9 ± 1.8	38.2 ± 9.1	71.6 ± 11.4
Lactating dams[b]							
(Pup PND 1 to PND 14)	0	0.6 ± 0.2	3.5 ± 1.0	14.0 ± 4.7	37.3 ± 11.5	84.0 ± 34.7	167.2 ± 49.9
Pups after weaning							
(PND 21 to PND 50)							
Males	0	0.6 ± 0.1	3.0 ± 0.4	10.8 ± 2.1	28.7 ± 4.8	68.9 ± 10.1	166.4 ± 49.7
Females	0	0.6 ± 0.1	2.9 ± 0.4	11.7 ± 1.9	30.5 ± 4.7	72.7 ± 9.9	166.1 ± 32.9

[a] Data are presented as mg genistein/kg body weight per day ± standard deviation
[b] Data used for calculation of ingested dose during the lactation period (PND 1 to 21) were limited to PND 1 to 14 because of significant direct consumption of feed by pups between PNDs 14 and 21.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF GENISTEIN

Genistein for this study was obtained from Toronto Research Chemicals, Inc. (North York, Ontario, Canada), in one lot (1-BP-118-3). Identity and purity analyses were conducted by the study laboratory, the National Center for Toxicological Research (NCTR), Jefferson, AR (Appendix A). Reports on analyses performed in support of the reproductive dose range-finding study of genistein are on file at the NCTR.

The chemical, a pale-yellow solid, was identified as genistein using mass spectrometry and ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy. The purity of lot 1-BP-118-3 was determined using ^{1}H- and ^{13}C-NMR spectroscopy and high-performance liquid chromatography (HPLC). Both ^{1}H- and ^{13}C-NMR spectroscopy results indicated the presence of a single impurity (1.1%, mole/mole) containing an ethyl group that was tentatively identified as ethanol. The purity profile obtained using HPLC indicated a purity of at least 99%. The overall purity was determined to be 99% or greater.

To ensure stability, the bulk chemical was stored at –70° C, protected from light, in opaque white plastic containers provided by the chemical manufacturer.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared approximately every 1 to 3 weeks by mixing genistein with soy- and alfalfa-free Purina 5K96 feed (Table A1). The specifications for this diet can be found in the multigenerational reproductive toxicology and chronic genistein Technical Reports (NTP, 2007, 2008c). Homogeneity and stability studies of the 5 ppm dose formulation were performed by the study laboratory using HPLC. Homogeneity was confirmed, and the stability of the dose formulations was confirmed for at least 22 days stored at 4° ± 2° C in stainless steel cans protected from light.

During the reproductive dose range-finding study, the dose formulations were analyzed five times by the study laboratory using HPLC. All 13 dose formulations analyzed were within 10% of the target concentrations (Table A2).

REPRODUCTIVE DOSE RANGE-FINDING STUDY

One week prior to breeding to untreated F_0 males, 70- to 80-day-old F_0 female Sprague-Dawley rats from the study laboratory's breeding colony were shifted from the standard NIH-31 pellet diet to the soy- and alfalfa-free Purina 5K96 meal diet. The specifications for this diet can be found in NTP, 2007 and 2008c. The NIH-31 diet has been reported to contain approximately 30 ppm of both genistein and daidzein (Thigpen *et al.*, 1999). Serum levels in the range of 0.3 to 0.6 µM have been measured in rats consuming this diet (Chang and Doerge, 2000). The parental generation in this study was raised on NIH-31 diet and was shifted to the soy- and alfalfa-free 5K96 diet prior to breeding. Because the half-life of genistein in serum is approximately 4 hours in female rats (Chang *et al.*, 2000), the 1-week washout period was sufficient to clear genistein from the circulation. Two animals per week from this colony were sent for microbiological surveillance to ensure that the colony was free of parasites and bacterial pathogens. Vaginal plug-positive females were assigned to the study and housed individually.

On gestation day 6 (GD 6, plug date=day 0), 10 untreated dams were randomly assigned to each exposure group to ensure that five litters would be obtained for each exposure concentration. Administration of dosed feed (0, 5, 25, 100, 250, 625, or 1,250 ppm genistein) was started on GD 7, and dams were continued on the same diets through weaning of their litters on postnatal day 21 (PND 21). Pregnant females were observed twice daily from GD 7 until parturition, and any signs of abnormal appearance or behavior were recorded. During this period, feed consumption and body weights were recorded daily.

The day of birth was designated as PND 1, and gestation duration was calculated from this date. Data on litter production, length of gestation, litter parameters, and pup anogenital distance (AGD) were collected and are reported on all litters produced, but only five litters per exposure group were randomly selected for further evaluation. Blood samples were collected from two control dams whose litters were not used, one at 1 month and one at study termination, for microbiological surveillance according to the protocols of the study laboratory's Sentinel Animal Program. The sera were analyzed for antibody titers to rodent viruses, and all sentinel animals were examined for ectoparasites, endoparasites, and bacterial pathogens. All results were negative. At weaning, all other dams were euthanized and not otherwise evaluated. Statistical analysis of dam body weight and feed consumption during pregnancy was limited to the dams producing the five litters kept on the study, except for the control group where the data from two dams used for microbiological surveillance were also included.

On PND 1, the number of live and dead pups, litter weight (live pups), sex ratio, and any gross malformations were recorded for the F_1 animals. On PND 2, the pups were weighed, litters were randomly standardized to four males and four females each, AGD was measured on all pups with an ocular micrometer, and the pups were identified by paw tattoo. During litter randomization, littermates were kept together. Pups were fostered within exposure groups

when necessary; however, this was rare (a total of five males, one each in the 0, 25, and 625 ppm exposure groups and two in the 1,250 ppm group), and none of the reported necropsy data are from fostered pups. There was a shortage of pups in the 1,250 ppm group, in that one litter had only six pups (three/sex). A litter mean AGD for each sex was calculated from three measurements made on each pup by a reader blind to exposure group. Pups were monitored daily for developmental landmarks, including day of eye opening, incisor eruption, ear unfolding, fur development, and timed righting reflex.

On PNDs 4, 7, 14, and 21, body weights and number of pups alive and dead were recorded. At weaning on PND 21, the pups were individually identified by tail tattoos, housed in same sex pairs, and continued on the same dosed feed as their dams until the day prior to sacrifice on PND 50. Feed consumption and body weights of the pups were recorded weekly between PNDs 21 and 49. Starting on PND 21, female pups were monitored daily for vaginal opening, and male pups were examined for preputial separation and testicular descent. Feed and filtered tap water were provided *ad libitum* throughout the experiment. Additional details of the study design and animal maintenance are summarized in Table 2.

Necropsies were performed on three male and three female pups per litter; the fourth pup of each sex in each litter was removed and used for the neuroanatomical studies reported in Appendix B. Animals were fasted overnight prior to weighing, euthanasia, and necropsy on the following morning, PND 50. Three animals of each sex from each litter were examined for organ weights and histopathology. At necropsy, carcasses were examined for gross lesions, and lesions and protocol-specified tissues were processed for microscopic evaluation. Reproductive organs, accessory reproductive organs, and mammary glands were examined in all exposure groups; all other protocol-specified tissues were examined in the 0 and 1,250 ppm groups. If an increase in incidence or severity of lesions was detected microscopically in any of the specified tissues of the 1,250 ppm animals, those tissues were examined in all the animals from the intermediate exposure concentration groups. For females, the ovary/oviduct, uterus, and vagina were weighed separately, fixed in Bouin's solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for histologic evaluation. For males, the testis and epididymis were weighed. The right testis and epididymis were used for determination of homogenization-resistant spermatids and for sperm analysis, respectively (Robb *et al.,* 1978). The left testis and epididymis were fixed in Bouin's solution and embedded in paraffin. The fixed and embedded testes were sectioned and stained with periodic acid-Schiff/hematoxylin to detect different stages of the seminiferous tubules. Testes sections did not include the rete testis. Seminal vesicles with coagulating and preputial glands were weighed and fixed in 10% neutral buffered formalin (NBF). The prostate gland was fixed in NBF, and the dorsolateral and ventral lobes were dissected and weighed separately. For both sexes, adrenal gland, bladder, heart, kidney, liver, lung, pituitary gland, spleen, thymus, thyroid gland, ureter, and urethra were collected. The liver, spleen, and thymus were weighed, fixed in NBF, embedded in paraffin, sectioned, and stained with H&E.

Pituitary and thyroid glands were weighed after fixation in NBF and then processed for histopathologic evaluation. Adrenal gland, heart, kidney, lung, and urinary tract were fixed in NBF and processed for histopathologic evaluation. The third left abdominal mammary gland was prepared as a whole mount fixed in NBF and stained with alum carmine for qualitative assessment of terminal end buds, terminal ducts, alveolar buds, and lobules. The corresponding mammary gland from the right side was fixed in NBF, embedded in paraffin, and stained with H&E for histologic evaluation. The right femur was removed, measured, and fixed in NBF. After decalcification, a cross-section at exactly mid-shaft was stained with H&E. Bone marrow from the sternum was evaluated histologically. For all tissues, sectioning was conducted at 4 to 6 µm.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the study laboratory's Micropath Data Collection System. The slides, paraffin blocks, and residual wet tissues were sent to the study laboratory's Block and Slide Laboratory for inventory, slide/block match, and wet tissue audit.

TABLE 2
Experimental Design and Materials and Methods in the Reproductive Dose Range-Finding Feed Study of Genistein

Study Laboratory
National Center for Toxicological Research (NCTR) (Jefferson, AR)

Strain and Species
Sprague-Dawley/CD23/Nctr BR rats

Animal Source
NCTR breeding colony (Jefferson, AR)

Average Age When Study Began
Gestational day 7 (GD 7)

Date of First Exposure
June 30-July 2, 1997

Duration of Exposure
64 days (GD 7 through PND 49)

Date of Last Exposure
September 2-4, 1997

Necropsy Dates
September 3-5, 1997

Average Age at Necropsy
50 days

Size of Study Groups
5 litters consisting of 4 male and 4 female pups

Method of Distribution
Vaginal plug-positive dams were randomly assigned to dose groups on GD 6; litters were randomly culled to 8 (4 males and 4 females) on PND 2.

Animals per Cage
Pregnant dams were housed individually. Pups were kept with their mothers and then were housed in same sex pairs after weaning on PND 21.

Method of Animal Identification
Paw tattoo, tail tattoo

Diet
Purina 5K96 rodent chow, irradiated (Test Diets, Purina Mills, Richmond, IN), available *ad libitum*

Water
Millipore-filtered tap water (Jefferson municipal supply) via water bottle, available *ad libitum*

Cages
Solid-bottom polycarbonate (Allentown Caging Equipment Co., Allentown, NJ), changed weekly

Bedding
Heat-treated hardwood chips (P.J. Murphy Forest Products, Inc., Montville, NJ), changed weekly

Cage Bonnets
Microisolator tops (Lab Products, Inc., Maywood, NJ)

TABLE 2
Experimental Design and Materials and Methods in the Reproductive Dose Range-Finding Feed Study of Genistein

Racks
Stainless steel (Allentown Caging Equipment Co., Allentown, NJ), changed every 28 days

Animal Room Environment
Temperature: 73° ± 5° F
Relative humidity: 50% ± 20%
Room fluorescent light: 12 hours/day
Room air changes: at least 10/hour

Exposure Concentrations
0, 5, 25, 100, 250, 625, or 1,250 ppm in feed, available *ad libitum*

Type and Frequency of Observation
From GD 7 until parturition, the dams were observed twice daily, and their daily body weights and feed consumption were recorded. Reproductive performance of the dams was recorded at parturition. Dams were sacrificed without further analysis when the pups were weaned on PND 21. Pups were observed twice daily and weighed on PNDs 2, 4, 7, 14, and 21, weekly until PND 49, and at sacrifice on PND 50. Clinical findings were recorded weekly, and feed consumption was measured weekly from PND 21 to 49. Reproductive and developmental endpoints were recorded at various time points from PNDs 1 to 49.

Method of Sacrifice
For two pups/sex per litter: anesthetized with carbon dioxide/oxygen, bled by cardiac puncture, and euthanized with carbon dioxide, following overnight fasting with water only. For one pup/sex per litter: decapitation, following overnight fasting with water only. (The brain tissue from these animals was transferred to the NCTR Division of Neurotoxicology for studies not reported here). The fourth pup of each sex was overdosed with sodium pentobarbital and then perfused transcardially with 0.9% saline followed by 10% buffered formalin. The brain was then prepared for three-dimensional reconstruction and volume measurement as described in Appendix B.

Necropsy
Necropsies were performed on three pups/sex per litter. Organs weighed were the coagulating gland, epididymis, liver, left and right ovary/oviduct, pituitary gland, preputial gland, dorsolateral and ventral prostate gland, seminal vesicle, spleen, left and right testis, thymus, thyroid gland, uterus, and vagina.

Histopathology
Complete histopathology was performed on pups in the 0 and 1,250 ppm groups. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, clitoral gland, coagulating gland, heart, kidney, liver, lung, mammary gland, ovary, oviduct, pituitary gland, preputial gland, dorsolateral and ventral prostate gland, spleen, left testis with epididymis and seminal vesicle, thymus, thyroid gland, ureter, urinary bladder, uterus, and vagina. Reproductive organs, accessory sex organs, and mammary gland were examined in the remaining exposed groups; other organs were examined in the remaining exposed groups if lesions were noted in the 1,250 ppm group.

Sperm Count
At the end of the study, sperm samples were collected from the right testis and epididymis of male pups in all exposure groups for spermatid head counts and epididymal spermatozoa counts.

STATISTICAL METHODS

For the dams, daily body weights and feed consumption during pregnancy were analyzed by repeated measures ANOVA using a mixed model approach. Dunnett's (1955) test was used to make comparisons between control and genistein-exposed groups, and contrasts were used to test for linear exposure concentration trends at each time interval.

For the pups, body weight gain and total feed consumption data were analyzed by split-plot ANOVA, and daily body weights were analyzed by repeated measures ANOVA with a split-plot error structure and split-plot analyses at each time point. The litter was the whole plot factor, and the subsets of same sex pups in the litter were the subplot factors. Average male and average female responses per litter were the endpoints analyzed. Two-sided Dunnett's tests were used to make comparisons between control and exposed groups. Because there was a small number of fostered pups, separate analyses were conducted using nursing dam and birth dam as the litter identifier. Conclusions were identical for both analyses, and reported results are based on the nursing dam as the litter identifier. Pup organ weights and measures of sexual maturation (vaginal opening and preputial separation) were analyzed separately by sex using a nested mixed model ANOVA that contained exposure concentration as a fixed factor and nursing dam nested within exposure concentration and residual error as random factors. For organ weights, tests for linear and quadratic exposure-concentration trends were conducted using contrasts and, for both organ weights and markers of sexual maturation, two-sided Dunnett's tests were used to compare means of genistein-exposed groups to the means of the control group. Markers of development were similarly analyzed. Litter size, mean live pup weight, percent male pups, gestation duration, ovarian follicle counts, testicular spermatid head counts, and epididymal spermatozoa counts were analyzed by one-way ANOVA and Dunnett's test. Analysis of covariance was used to analyze mean live pup weight and litter mean anogenital distance, with litter size and mean pup weight, respectively, designated as covariates. Anogenital distance was analyzed separately by sex. The proportions of dams producing litters and of stillborn pups were analyzed using an exact Chi-square test.

Histopathology data were analyzed for genistein effects on lesion incidences and severities by the Jonckheere-Terpstra test (Hollander and Wolfe, 1973). Williams' modification of Shirley's test (Williams, 1986) was used to compare exposed groups to the control group. Estrous cycle stage synchrony between the uterus and vagina was analyzed in a similar manner. All statistical tests were conducted at the α=0.05 level.

QUALITY ASSURANCE METHODS

This study was conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). The Quality Assurance Unit of the NCTR performed audits and inspections of protocols,

procedures, data, and reports throughout the course of the studies. Separate audits covering completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Toxicity Study Report were conducted. Audit procedures and findings are presented in the reports and are on file at the NCTR. The audit findings were reviewed and assessed by NCTR staff, and all comments were resolved or otherwise addressed during the preparation of this Toxicity Study Report.

RESULTS

REPRODUCTIVE DOSE RANGE-FINDING STUDY

Body Weights and Feed Consumption of Dams During Pregnancy

Body weights and feed consumption of the dams during pregnancy are shown in Figures 2 and 3, respectively. Body weight gain during pregnancy was affected by genistein only in the 1,250 ppm group. Although there was not a strictly linear decrease in body weights with exposure concentration, a linear trend test was significant for gestational days (GDs) 12 to 21 (Table 3). In pairwise comparisons with the control group, the mean body weights of the 1,250 ppm group were significantly less than those of the controls on GDs 20 and 21. The 1,250 ppm group mean feed consumption (Figure 3) was significantly less than that by the control group. Total body weight gain and pooled feed consumption from GDs 8 through 21 of the 1,250 ppm group were significantly less than the totals seen in the control group (66% and 80% of the control group values, respectively; Tables 3 and 4).

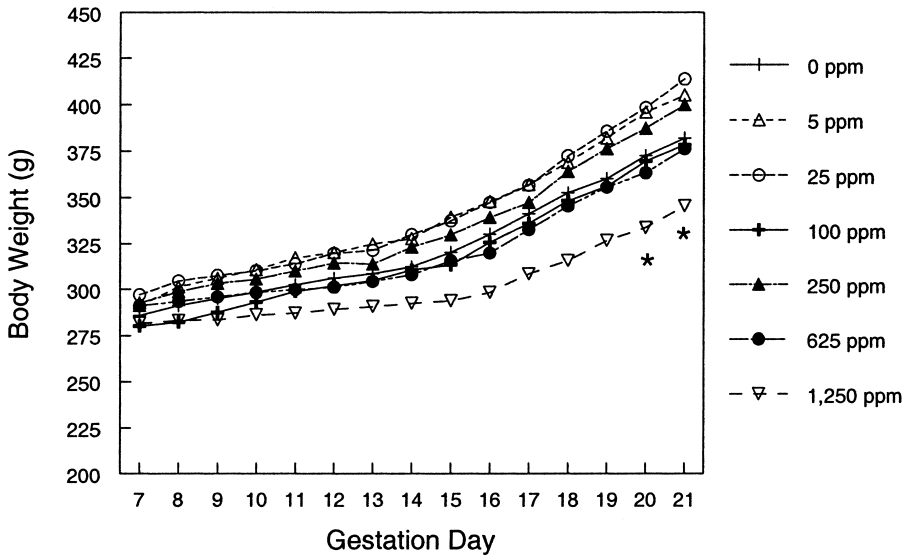


FIGURE 2
Body Weights of Dams During Pregnancy in the Reproductive Dose Range-Finding Feed Study of Genistein
n=5 for all groups except the controls, where n=7. Data from the five dams from each exposed group whose litters were kept on the study were analyzed. For the controls, data from two additional dams that produced litters that were used as microbiological sentinels were included.

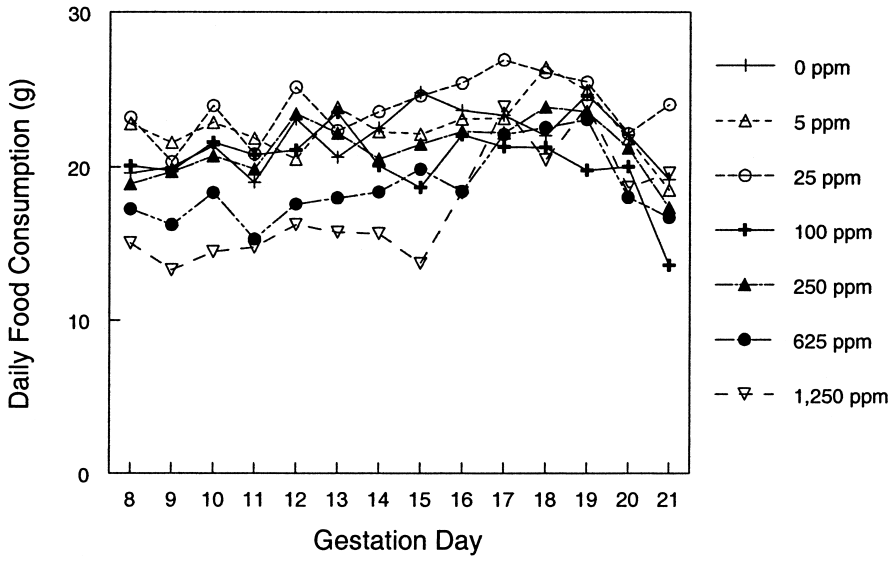


FIGURE 3
Feed Consumption of Dams During Pregnancy in the Reproductive Dose Range-Finding Feed Study of Genistein
n=5 dams per exposure group except for the 625 ppm group on gestation day 17, where n=4, and the controls, where n=7

TABLE 3
Body Weights of Dams During Pregnancy in the Reproductive Dose Range-Finding Feed Study of Genistein[a]

Gestation Day[b]	0 ppm	5 ppm	25 ppm	100 ppm	250 ppm	625 ppm	1,250 ppm
7	286 ± 6	291 ± 4	297 ± 11	280 ± 6	293 ± 12	291 ± 9	282 ± 10
8	291 ± 7	301 ± 5	305 ± 12	282 ± 6	299 ± 11	294 ± 10	283 ± 8
9	295 ± 8	306 ± 4	308 ± 10	287 ± 5	303 ± 12	296 ± 10	284 ± 8
10	299 ± 8	311 ± 4	310 ± 12	293 ± 6	306 ± 12	298 ± 9	286 ± 8
11	302 ± 8	317 ± 4	314 ± 12	299 ± 6	310 ± 13	300 ± 9	287 ± 8
12[c]	306 ± 8	320 ± 3	320 ± 13	302 ± 6	315 ± 12	301 ± 9	289 ± 8
13[c]	309 ± 9	325 ± 4	321 ± 13	305 ± 6	314 ± 13	304 ± 10	291 ± 7
14[c]	312 ± 9	328 ± 4	330 ± 12	311 ± 6	323 ± 12	308 ± 10	293 ± 9
15[c]	320 ± 10	339 ± 4	338 ± 13	313 ± 6	330 ± 11	316 ± 10	294 ± 9
16[c]	330 ± 10	348 ± 4	347 ± 13	326 ± 6	339 ± 12	320 ± 10	298 ± 6
17[c]	341 ± 10	357 ± 4	357 ± 12	336 ± 4	347 ± 12	333 ± 9	309 ± 6
18[c]	353 ± 11	369 ± 4	373 ± 12	348 ± 7	364 ± 12	346 ± 12	316 ± 6
19[c]	360 ± 13	382 ± 5	386 ± 14	356 ± 6	376 ± 13	356 ± 10	327 ± 6
20[c]	373 ± 14	396 ± 5	398 ± 13	370 ± 8	387 ± 15	363 ± 11	334 ± 5*
21[c]	382 ± 15	405 ± 5	414 ± 14	378 ± 7	400 ± 15	376 ± 12	346 ± 2*

* Significantly different ($P \leq 0.05$) from the control group by Dunnett's test
[a] n=5 in all groups except the control group, where n=7
[b] GD 0 is the first day a dam was observed to be vaginal plug positive
[c] Significant ($P \leq 0.05$) linear dose trend as determined by contrasts

TABLE 4
Feed Consumption of Dams During Pregnancy in the Reproductive Dose Range-Finding Feed Study of Genistein[a]

	0 ppm	5 ppm	25 ppm	100 ppm	250 ppm	625 ppm	1,250 ppm
Feed consumption (g)	306 ± 14	316 ± 10	334 ± 14	284 ± 10	297 ± 11	275 ± 21	244 ± 12*

* Significantly different ($P \leq 0.05$) from the control group by Dunnett's test
[a] n=5 in all groups except the control group, where n=7

Effects of Genistein on Litter Production, Gestation Duration, Litter Parameters

There was an apparent treatment effect on the proportion of vaginal plug-positive dams that delivered litters (Chi-square, $P<0.05$), with only five of 10 animals in the 1,250 ppm group producing litters compared with eight to 10 litters produced in all other groups (Table 5). Genistein exposure was not significantly related to gestation duration, litter size, proportion of dead pups, or sex ratio. Mean live pup body weight was lower in the 1,250 ppm group than in the controls, but while an analysis of covariance, with litter size as the covariate, indicated a significant exposure concentration effect, comparison of each exposed group to the controls by Dunnett's test indicated that the mean pup body weight of the 1,250 ppm group was not significantly different from that of the controls ($P=0.07$).

Effects of Dietary Genistein on Anogenital Distance, Pup Developmental Landmarks, and Body Weight

Genistein had no apparent effect on anogenital distance measured on postnatal day (PND) 2 in either sex (Table 5). There were no significant exposure-related effects on landmarks of sexual development (age of testicular descent and preputial separation in males and age of vaginal opening in females); although the time of vaginal opening in the 1,250 ppm group was approximately 2.9 days earlier than that in the controls (Table 6), this difference was not significant ($P=0.08$). Since the means in the 1,250 ppm groups for multiple markers of development appeared to differ from those in the controls, litter means were analyzed by one-way ANOVA. Significant effects of exposure concentration and linear exposure concentration trends toward delay were observed for righting reflex, eye opening, and ear unfolding in males, and for eye opening and ear unfolding in females (Table 6). Pairwise comparisons indicated that the 1,250 ppm groups were significantly different from the controls for eye opening and ear unfolding in males and females, and the 625 ppm group was significantly different from the control group in the case of the righting reflex in females. No significant differences in the timing of the appearance of fur were observed (Table 6). The delay observed in these endpoints may be related to the lower body weights of the pups in the 1,250 ppm groups, particularly since means for nearly all endpoints were delayed in the 1,250 ppm groups, although not significantly so in all cases.

The mean body weights of male and female pups are shown in Figure 4. The weekly mean body weights of the 1,250 ppm groups were significantly less than those of the control groups at PND 14 and at all subsequent time points through PND 49. The total body weight gains of male and female pups from birth to termination of the study are shown in Table 7. Genistein affected total body weight gain in a similar manner for both sexes, with weight gains in the 1,250 ppm groups lower than those in the controls.

TABLE 5
Litter Data for Rats in the Reproductive Dose Range-Finding Feed Study of Genistein

	0 ppm	5 ppm	25 ppm	100 ppm	250 ppm	625 ppm	1,250 ppm
Litters/plug-positive females[a]	9/10	9/10	10/10	9/10	10/10	8/10	5/10
Gestation duration (days)[b,c]	22.3 ± 0.3	22.8 ± 0.1	22.7 ± 0.2	22.9 ± 0.1	22.6 ± 0.2	22.0 ± 0.0	22.4 ± 0.2
Total pups/litter[b]	10.6 ± 1.6	14.4 ± 0.8	11.6 ± 1.0	13.8 ± 1.0	12.2 ± 1.1	12.0 ± 1.1	10.4 ± 1.3
Stillborn pups/ total pups	3/95	4/130	0/116	2/124	0/122	3/96	0/52
Male pups (%)[b]	48.5 ± 7.7	49.3 ± 4.4	56.9 ± 4.9	53.8 ± 3.9	57.2 ± 5.7	52.3 ± 5.3	42.3 ± 5.8
Mean live pup weight (g)[b,c]	6.79 ± 0.26	6.27 ± 0.17	6.74 ± 0.16	6.47 ± 0.23	6.45 ± 0.25	6.29 ± 0.20	6.14 ± 0.60
Male anogenital distance (mm)[c,d]							
n	9	5	8	6	8	6	5
	3.58 ± 0.20	3.30 ± 0.02	3.58 ± 0.13	3.69 ± 0.19	3.40 ± 0.06	3.30 ± 0.08	3.17 ± 0.15
Female anogenital distance (mm)[c,d]							
n	8	5	8	6	8	6	5
	2.13 ± 0.13	2.03 ± 0.15	1.94 ± 0.03	2.30 ± 0.18	2.17 ± 0.16	2.05 ± 0.15	2.02 ± 0.10

[a] Significant effect of exposure concentration indicated by exact Chi-square test ($P<0.05$)
[b] Mean ± standard error, data from all litters born are included
[c] Significant main effect of exposure concentration ($P<0.05$)
[d] Litter means ± standard error are presented for all litters in which anogenital distance (AGD) was measured. Plugged dams were delivered to the study over a 2-week period from the NCTR breeding colony. The dams were randomly allocated to exposure groups on arrival, and approximately 80% of the dams were expected to litter. Since it was not known which of the allocated dams would litter and become part of the five litters per exposure group kept on study, the AGD of each pup in each litter that could potentially become assigned to the study was measured. The five litter positions per exposure group were filled with the first available litters that did not contain fostered pups or have an inattentive dam. If a litter was born to an exposure group that already had five litters assigned, AGD was not measured. One litter in the control group had all males, thus accounting for the difference in litter number for males and females in that exposure group.

TABLE 6
Developmental Landmarks in Rat Pups in the Reproductive Dose Range-Finding Feed Study of Genistein[a]

	0 ppm	5 ppm	25 ppm	100 ppm	250 ppm	625 ppm	1,250 ppm
Male							
Righting reflex[b,c]	1.80 ± 0.41	2.00 ± 0.35	1.50 ± 0.32	1.00 ± 0.21	2.10 ± 0.42	2.75 ± 0.28	2.40 ± 0.27
Fur development	8.10 ± 0.71	9.10 ± 0.57	9.70 ± 0.44	9.00 ± 0.32	8.75 ± 0.46	9.50 ± 0.63	8.60 ± 0.95
Eye opening[b,c]	16.35 ± 0.38	16.40 ± 0.24	16.30 ± 0.49	16.35 ± 0.33	15.60 ± 0.51	17.50 ± 0.31	18.00 ± 0.45[d]
Ear unfolding[b,c]	17.60 ± 0.60	17.80 ± 0.37	17.47 ± 0.49	18.20 ± 0.20	17.40 ± 0.24	18.60 ± 0.24	19.40 ± 0.51[d]
Incisor eruption[c]	10.20 ± 0.46	10.75 ± 0.34	10.85 ± 0.33	10.40 ± 0.40	10.60 ± 0.30	11.21 ± 0.28	11.32 ± 0.34
Testicular descent	22.50 ± 0.43	22.00 ± 0.43	22.42 ± 0.32	22.20 ± 0.37	21.80 ± 0.50	22.90 ± 0.10	22.85 ± 0.58
Preputial separation	42.20 ± 0.27	42.42 ± 0.21	41.38 ± 0.58	41.45 ± 0.32	42.20 ± 0.32	42.94 ± 0.37	43.53 ± 0.41
Female							
Righting reflex[b]	2.05 ± 0.62	2.47 ± 0.35	2.00 ± 0.33	1.70 ± 0.27	2.90 ± 0.30	3.70 ± 0.36[d]	2.57 ± 0.39
Fur development	7.70 ± 0.54	9.20 ± 0.63	9.60 ± 0.40	9.05 ± 0.28	9.20 ± 0.37	9.50 ± 0.67	8.50 ± 0.98
Eye opening[b,c]	16.30 ± 0.37	16.40 ± 0.24	16.20 ± 0.49	16.20 ± 0.37	15.60 ± 0.51	17.60 ± 0.24	18.10 ± 0.40[d]
Ear unfolding[b,c]	17.60 ± 0.60	17.80 ± 0.37	17.50 ± 0.39	18.20 ± 0.20	17.40 ± 0.24	18.60 ± 0.24	19.27 ± 0.41[d]
Incisor eruption	10.00 ± 0.45	11.00 ± 0.48	10.65 ± 0.33	10.60 ± 0.29	10.55 ± 0.44	11.40 ± 0.20	11.18 ± 0.22
Vaginal opening	33.25 ± 0.32	32.05 ± 0.55	33.30 ± 0.60	32.55 ± 0.44	31.60 ± 0.37	32.60 ± 0.69	30.33 ± 0.50

[a] Postnatal day; litter mean ± standard error (n=5)
[b] Significant main effect of exposure concentration (P<0.05)
[c] Significant linear exposure concentration trend (P<0.05)
[d] Significantly different (P<0.05) from the control group by Dunnett's test

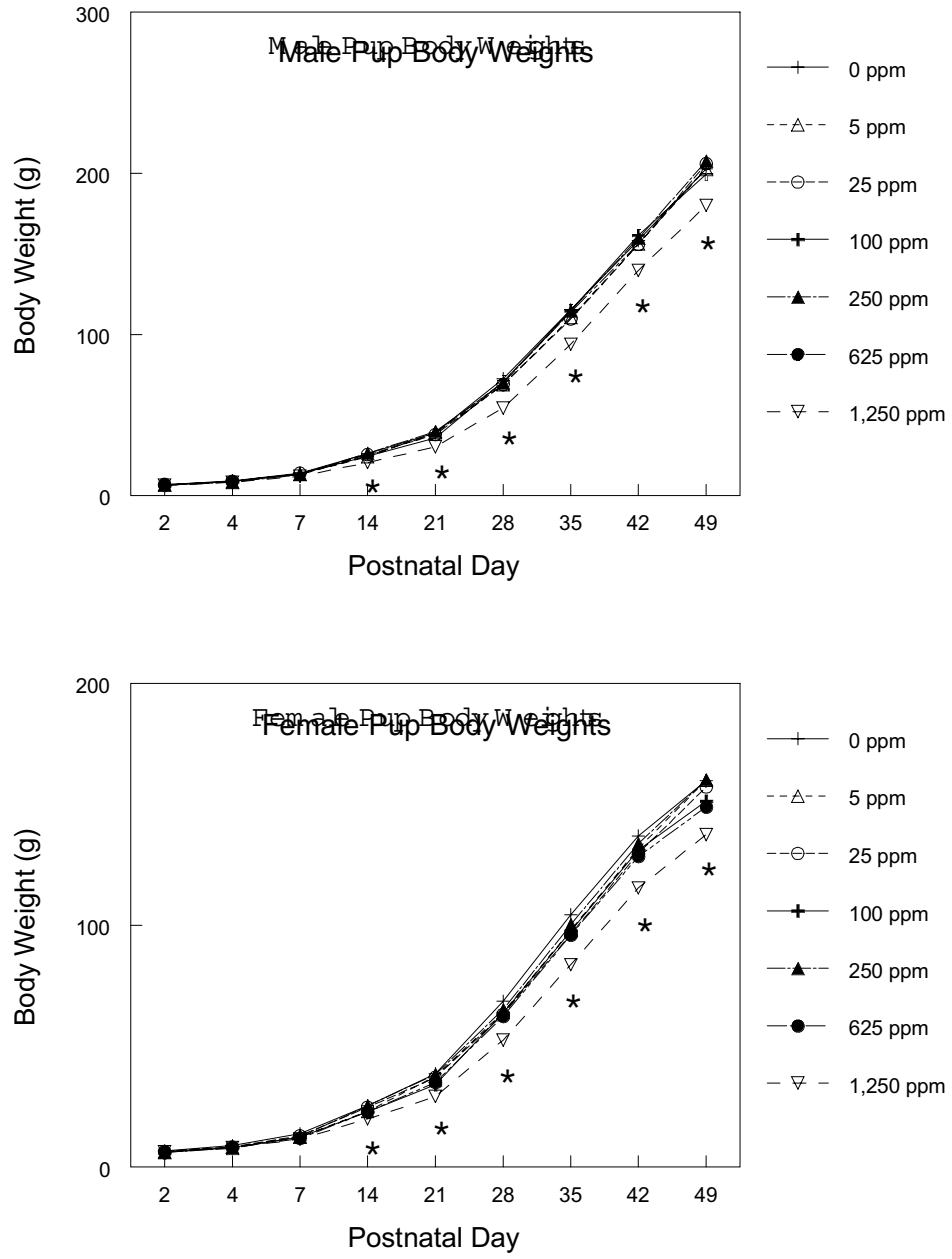


FIGURE 4
Body Weights of Rat Pups
in the Reproductive Dose Range-Finding Feed Study of Genistein
Generally n=20 pups per exposure group

TABLE 7
Total Body Weight Gains of Rat Pups in the Reproductive Dose Range-Finding Feed Study of Genistein[a]

	0 ppm	5 ppm	25 ppm	100 ppm	250 ppm	625 ppm	1,250 ppm
Male							
n	20	20	19	20	20	20	17
	196 ± 2	196 ± 2	199 ± 3	193 ± 3	201 ± 4	197 ± 5	174 ± 4*
Female							
n	20	20	20	20	20	20	18
	153 ± 3	153 ± 3	151 ± 2	145 ± 2	153 ± 2	143 ± 2	132 ± 2*

* Significantly different (P<0.05) from the control group by Dunnett's test
[a] Weights are given in grams as mean ± standard error. Pup body weight gains (from birth to study termination) were analyzed using a split-plot model with nursing litter as the whole-plot unit, the exposure concentration of genistein as the whole-plot factor, and the sex of the pup as the split-plot factor. The response was fitted with the fixed effects of exposure concentration, sex, and an exposure concentration × sex interaction. The variance was fitted with the random effects litter dam nested within exposure concentration and a residual error term. The exposure concentration and sex main effects were significant (P=0.0004 and 0.0001, respectively), but the exposure concentration × sex interaction was not (P=0.54). Since the exposure concentration × sex interaction was not significant, the exposure concentrations were compared independently of sex. For the pooled sexes, the test for linear trend was significant (P=0.0001), although body weight gain did not strictly decrease with exposure concentration. When this analysis was rerun without the 1,250 ppm group, the linear trend was no longer significant (P=0.33).

Effects of Genistein on Terminal Body Weights and Absolute and Relative Organ Weights

Males

The mean terminal body weight of 1,250 ppm male pups was 10% less than that of the controls (Table 8). Absolute liver weight was significantly increased in the 250 ppm group (Table 8). Relative liver weight was significantly increased in the 1,250 ppm group, but absolute liver weight was not increased in this group, and this effect appeared to be secondary to the decreased body weight noted above. The absolute thymus weight of 1,250 ppm males was 22% less than that of the controls, but the relative weight of the thymus in this group was not significantly affected. Absolute and relative ventral prostate gland weights were significantly decreased in the 1,250 ppm group (28% and 20%, respectively), and relative pituitary gland weight was significantly increased in this exposed group. No significant quadratic trend components were detected. Mean weights of the dorsolateral prostate gland were generally increased in the exposed groups, and those of the ventral prostate gland were increased in the 100 ppm group; none of these increases were statistically significant.

Females

The mean terminal body weight of 1,250 ppm female pups was 12% less than that of the controls (Table 9). In females, a significant positive trend was observed for relative pituitary gland weights (Table 9). Pairwise comparisons using Dunnett's test showed a significantly increased relative pituitary gland weight (19%) in the

TABLE 8
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Male Rat Pups in the Reproductive Dose Range-Finding Feed Study of Genistein[a]

	0 ppm	5 ppm	25 ppm	100 ppm	250 ppm	625 ppm	1,250 ppm
n	15	15	15	15	15	15	15
Necropsy body wt	188.23 ± 1.86	187.55 ± 3.25	194.31 ± 4.20	184.89 ± 2.27	197.10 ± 3.93	190.01 ± 5.71	170.56 ± 4.04*
Epididymis							
Absolute	0.272 ± 0.010	0.285 ± 0.009	0.286 ± 0.008	0.290 ± 0.006	0.283 ± 0.008	0.286 ± 0.012	0.269 ± 0.008
Relative	0.145 ± 0.006	0.152 ± 0.005	0.148 ± 0.004	0.157 ± 0.003	0.144 ± 0.004	0.150 ± 0.003	0.158 ± 0.003
Liver							
Absolute[b]	6.660 ± 0.136	7.070 ± 0.183	7.164 ± 0.161	6.594 ± 0.140	7.470 ± 0.202*	6.743 ± 0.210	6.605 ± 0.218
Relative[b]	3.533 ± 0.062	3.780 ± 0.105	3.696 ± 0.075	3.565 ± 0.055	3.793 ± 0.076	3.552 ± 0.058	3.868 ± 0.081*
Pituitary Gland							
Absolute[c]	9.1 ± 0.4	9.1 ± 0.3	9.0 ± 0.4	9.2 ± 0.3	10.1 ± 0.5	9.8 ± 0.5	10.1 ± 0.3
Relative[b,c]	0.0048 ± 0.0002	0.0048 ± 0.0002	0.0046 ± 0.0002	0.0050 ± 0.0002	0.0051 ± 0.0002	0.0051 ± 0.0002	0.0060 ± 0.0002*
Preputial Gland							
Absolute	93.1 ± 7.4	79.6 ± 6.1	70.6 ± 5.8	84.5 ± 5.5	94.9 ± 3.7	98.1 ± 3.1	94.3 ± 6.0
Relative[b,c]	0.050 ± 0.004	0.042 ± 0.003	0.037 ± 0.003	0.046 ± 0.003	0.048 ± 0.002	0.052 ± 0.002	0.056 ± 0.004
Dorsolateral Prostate Gland							
Absolute	0.139 ± 0.007	0.148 ± 0.008	0.161 ± 0.007[d]	0.144 ± 0.006	0.154 ± 0.007	0.149 ± 0.010	0.142 ± 0.007
Relative	0.074 ± 0.004	0.079 ± 0.004	0.082 ± 0.003[d]	0.078 ± 0.003	0.078 ± 0.004	0.077 ± 0.004	0.083 ± 0.004
Ventral Prostate Gland							
Absolute[b,c]	0.158 ± 0.004	0.150 ± 0.009	0.155 ± 0.007[d]	0.173 ± 0.008	0.151 ± 0.008	0.137 ± 0.009	0.113 ± 0.003*
Relative[b,c]	0.084 ± 0.002	0.080 ± 0.006	0.079 ± 0.004[d]	0.093 ± 0.004	0.076 ± 0.004	0.071 ± 0.004	0.067 ± 0.002*
Seminal Vesicle/ Coagulating Gland							
Absolute	0.157 ± 0.013	0.141 ± 0.011	0.176 ± 0.014[d]	0.161 ± 0.015	0.172 ± 0.016	0.164 ± 0.014	0.155 ± 0.012
Relative	0.084 ± 0.007	0.075 ± 0.005	0.089 ± 0.006[d]	0.086 ± 0.007	0.086 ± 0.007	0.084 ± 0.007	0.090 ± 0.006
Spleen							
Absolute	0.528 ± 0.014	0.556 ± 0.016	0.589 ± 0.020	0.502 ± 0.018	0.615 ± 0.021	0.576 ± 0.028	0.544 ± 0.024
Relative	0.281 ± 0.007	0.298 ± 0.011	0.304 ± 0.008	0.272 ± 0.009	0.312 ± 0.008	0.302 ± 0.011	0.321 ± 0.016
L. and R. Testis							
Absolute	2.267 ± 0.059	2.204 ± 0.035	2.157 ± 0.054	2.239 ± 0.041	2.254 ± 0.044	2.212 ± 0.098	2.068 ± 0.065
Relative	1.208 ± 0.037	1.118 ± 0.029	1.114 ± 0.030	1.212 ± 0.023	1.146 ± 0.017	1.156 ± 0.033	1.211 ± 0.018
Thymus							
Absolute[b,c]	0.764 ± 0.023	0.736 ± 0.018	0.733 ± 0.034	0.663 ± 0.025	0.728 ± 0.028	0.656 ± 0.022	0.598 ± 0.024*
Relative	0.406 ± 0.013	0.393 ± 0.010	0.382 ± 0.025	0.359 ± 0.013	0.371 ± 0.016	0.351 ± 0.018	0.351 ± 0.012
Thyroid Gland							
Absolute	16.3 ± 0.9	15.2 ± 0.5	19.1 ± 1.4	19.1 ± 1.2	23.9 ± 2.7	21.6 ± 2.7	19.3 ± 2.5
Relative	0.0086 ± 0.0005	0.0082 ± 0.0004	0.0099 ± 0.0007	0.0103 ± 0.0006	0.0122 ± 0.0015	0.0113 ± 0.0012	0.0113 ± 0.0014*

* Significantly different (P<0.05) from the control group by Dunnett's test

[a] Mean ± standard error; the values given are based on individual pups. Organ weights were compared across the exposure groups using a mixed effects ANOVA with litter as a random factor to account for possible litter effects. Body weights and absolute weights of the epididymis, liver, dorsolateral and ventral prostate gland, seminal vesicle/coagulating gland, spleen, testes, and thymus are given in grams; absolute weights of the remaining organs are given in milligrams. Organ-weight-to-body-weight ratios (relative weights) are given as g organ weight/g body weight × 100.

[b] Significant main effect of exposure concentration (P<0.05)

[c] Significant linear exposure concentration trend (P<0.05)

[d] n=14

TABLE 9
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Female Rat Pups in the Reproductive Dose Range-Finding Feed Study of Genistein[a]

	0 ppm	5 ppm	25 ppm	100 ppm	250 ppm	625 ppm	1,250 ppm
n	15	15	15	15	15	15	15
Necropsy body wt	148.07 ± 2.68	147.59 ± 3.29	148.04 ± 2.83	143.35 ± 1.90	147.88 ± 2.68	139.50 ± 2.71*	130.55 ± 2.06*
Liver							
Absolute[b]	5.348 ± 0.154	5.304 ± 0.183	5.360 ± 0.132[e]	5.003 ± 0.154	5.540 ± 0.140	5.168 ± 0.111	4.982 ± 0.116
Relative[b]	3.614 ± 0.087	3.584 ± 0.062	3.629 ± 0.052[e]	3.483 ± 0.078	3.747 ± 0.068	3.715 ± 0.082	3.814 ± 0.055
L. and R. Ovary							
Absolute	87.4 ± 2.2	95.7 ± 4.7	88.7 ± 2.8	92.0 ± 3.7	91.2 ± 5.9	97.7 ± 4.5	83.3 ± 10.5
Relative	0.059 ± 0.002	0.065 ± 0.003	0.060 ± 0.002	0.064 ± 0.002	0.062 ± 0.004	0.071 ± 0.004	0.064 ± 0.008
Pituitary Gland							
Absolute[c,d]	9.9 ± 0.2	9.9 ± 0.2	8.9 ± 0.5	10.2 ± 0.5	10.9 ± 0.5	11.1 ± 0.4	9.7 ± 0.2
Relative[b,c,d]	0.0067 ± 0.0002	0.0068 ± 0.0002	0.0061 ± 0.0004	0.0071 ± 0.0003	0.0074 ± 0.0003	0.0080 ± 0.0002*	0.0074 ± 0.0002
Spleen							
Absolute	0.446 ± 0.014	0.458 ± 0.015	0.446 ± 0.012	0.385 ± 0.009	0.440 ± 0.020	0.418 ± 0.016	0.401 ± 0.014
Relative	0.302 ± 0.009	0.310 ± 0.008	0.302 ± 0.008	0.269 ± 0.005	0.297 ± 0.012	0.300 ± 0.011	0.308 ± 0.011
Thymus							
Absolute[b]	0.571 ± 0.016	0.634 ± 0.026	0.606 ± 0.033	0.583 ± 0.018	0.588 ± 0.014	0.556 ± 0.024	0.520 ± 0.023
Relative	0.387 ± 0.011	0.430 ± 0.015	0.411 ± 0.024	0.408 ± 0.014	0.400 ± 0.013	0.400 ± 0.018	0.398 ± 0.016
Thyroid Gland							
Absolute	15.7 ± 1.8	15.5 ± 0.8	17.9 ± 1.9	17.3 ± 1.3	21.1 ± 2.4	17.9 ± 1.2	19.3 ± 2.8
Relative	0.0106 ± 0.0011	0.0106 ± 0.0005	0.0121 ± 0.0013	0.0121 ± 0.0008	0.0141 ± 0.0015	0.0130 ± 0.0009	0.0149 ± 0.0023
Uterus							
Absolute[d]	0.334 ± 0.024	0.330 ± 0.032	0.290 ± 0.017	0.288 ± 0.018	0.314 ± 0.018	0.412 ± 0.047	0.294 ± 0.034
Relative[c,d]	0.228 ± 0.017	0.227 ± 0.022	0.196 ± 0.011	0.201 ± 0.012	0.212 ± 0.011	0.298 ± 0.035	0.224 ± 0.024
Vagina							
Absolute[b]	0.127 ± 0.007	0.130 ± 0.004	0.122 ± 0.006	0.116 ± 0.006	0.134 ± 0.006	0.127 ± 0.005	0.123 ± 0.007
Relative[b]	0.086 ± 0.005	0.088 ± 0.003	0.083 ± 0.004	0.081 ± 0.004	0.091 ± 0.004	0.091 ± 0.004	0.094 ± 0.006

* Significantly different (P<0.05) from the control group by Dunnett's test
[a] Mean ± standard error; the values given are based on individual pups. Organ weights were compared across the exposure groups using a mixed effects ANOVA with litter as a random factor to account for possible litter effects. Body weights and absolute weights of the liver, spleen, thymus, uterus, and vagina are given in grams; absolute weights of the remaining organs are given in milligrams. Organ-weight-to-body-weight ratios (relative weights) are given as g organ weight/g body weight × 100.
[b] Significant linear exposure concentration trend (P<0.05)
[c] Significant main effect of exposure concentration (P<0.05)
[d] Significant quadratic exposure concentration trend (P<0.05)
[e] n=14

625 ppm group. No significant differences in the weights of reproductive organs were noted between the exposed and control groups of females. Since females were in random cycle stages at the time of necropsy, only major effects on reproductive organ weights would have been detected.

Clinical Chemistry

Examination of the summary statistics of the clinical chemistry and hematology data collected indicated little or no effect of treatment. The thyroid stimulating hormone (TSH) values, however, showed considerable variation and a possible treatment effect. These data were further examined because of the known ability of isoflavones to inhibit

thyroid peroxidase activity and the fact that thyroid peroxidase activity in the animals from this study and similarly treated animals was shown to be depressed by genistein treatment (Chang and Doerge, 2000). No significant differences from the controls were observed in any exposed group for TSH or thyroid hormones, nor were lesions noted in the microscopic evaluation of the thyroid gland.

Histopathologic Examination of Male and Female Organs and Measures of Sperm Production

Males

No gross abnormalities, including retained or small testes, retention of Mullerian duct remnants, or hypospadias, were detected in male pups during necropsy. Exposure concentration-dependent increases in the incidences of minimal to mild hypertrophy and hyperplasia in mammary gland alveolar and duct epithelium were observed. The incidences of hypertrophy of the alveoli and ducts were significantly increased in groups exposed to 25 ppm or greater, and the incidences of alveolar hyperplasia were significantly increased in groups exposed to 250 ppm or greater (Table 10). Hyperplasia of the alveoli is defined as a relative increase of tubuloalveolar patterns of growth, characterized by alveoli attached to or in close approximation to branched, linear arrays of hypertrophied ducts. While the peripubertal age of the animals complicated the evaluation of spermatogenesis, there was evidence of exposure-related effects in the testis and epididymis. Seminiferous tubules showed retention of elongated spermatids in Stages X-XII and depletion of spermatids and degeneration of spermatocytes at earlier stages in the 1,250 ppm group. These effects were diagnosed as "abnormal spermatogenesis," and while this was observed in males in several exposure groups, including controls, the incidence of this lesion was significantly increased in the 1,250 ppm group. A significantly increased incidence of hypospermia in the head of the epididymis, consistent with the testicular effect, occurred in the 1,250 ppm group. One male in each of the 625 and 1,250 ppm groups had marked hypospermia (data not shown). In contrast to the histopathologic evaluation, testicular spermatid head counts and epididymal spermatozoa counts showed no statistically significant differences between the exposed and control groups (Table 11). A significant increase in the incidence of chronic inflammation of the dorsolateral prostate gland occurred in the 1,250 ppm group (Table 10). The inflammation was characterized by dense infiltration of lymphoid cells and more sparse infiltration of granulocytes into the stroma of the gland and occasional glandular alveoli. The incidence of depleted secretory fluid in the ventral prostate gland was significantly increased in 1,250 ppm males.

Outside of reproductive tract tissues and the mammary gland, the only other significant histopathologic effect of genistein exposure in males was renal tubule mineralization; incidences of this lesion were significantly increased in groups exposed to 250 ppm or greater (Table 10).

TABLE 10
Incidences of Selected Nonneoplastic Lesions in Male Rat Pups in the Reproductive Dose Range-Finding Feed Study of Genistein

	0 ppm	5 ppm	25 ppm	100 ppm	250 ppm	625 ppm	1,250 ppm
Mammary Gland[a]	13	13	14	15	15	14	13
Hypertrophy, Alveoli[b,d]	0	2 (2.0)[c]	4* (1.0)	4* (1.8)	7** (1.7)	9** (1.7)	8** (1.9)
Hypertrophy, Ducts[d]	0	2 (2.0)	5* (1.0)	4* (1.5)	6** (1.5)	9** (1.4)	8** (1.0)
Hyperplasia, Alveoli[d]	1 (1.0)	2 (2.0)	2 (1.0)	3 (1.7)	8** (1.6)	13** (1.7)	7** (1.7)
L. Testis	15	15	15	15	15	15	14
Abnormal Spermatogenesis[e]	2 (1.0)	0	3 (1.0)	0	2 (2.0)	2 (2.0)	7** (1.1)
L. Epididymis	15	15	15	15	14	15	14
Hypospermia[f]	3 (1.3)	5 (1.4)	4 (2.3)	3 (1.3)	3 (2.3)	7 (1.7)	9** (2.3)
Dorsolateral Prostate Gland	15	15	14	15	15	15	14
Inflammation[d]	6 (2.7)	3 (1.7)	4 (1.8)	3 (2.0)	4 (1.8)	9 (2.0)	12** (2.7)
Ventral Prostate Gland	15	15	14	15	15	15	14
Depletion of Secretory Fluid[e]	0	0	0	0	0	1 (4.0)	2* (2.0)
Kidney	15	15	15	15	15	15	14
Mineralization, Renal Tubule[d]	0	0	0	0	5** (1.2)	5** (1.2)	11** (1.5)

* Significantly different ($P<0.05$) from the control group by Shirley's test
** $P<0.01$
[a] Number of animals with tissue examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked
[d] Significant exposure concentration-related trend ($P<0.001$) by the Jonckheere-Terpstra test
[e] $P<0.05$
[f] $P<0.01$

TABLE 11
Testicular Spermatid Head Counts and Epididymal Sperm Counts in Male Rat Pups in the Reproductive Dose Range-Finding Feed Study of Genistein[a]

	0 ppm	5 ppm	25 ppm	100 ppm	250 ppm	625 ppm	1,250 ppm
n	7	12	10	11	9	7	7
R. Testicular Spermatid Head Counts (10^6)	64.84 ± 3.22	63.76 ± 3.03	57.10 ± 4.98	62.51 ± 2.59	59.86 ± 3.69	66.95 ± 4.44	61.22 ± 4.18
R. Epididymal Spermatocyte Counts (10^6)	52.14 ± 11.01	75.82 ± 5.95	62.61 ± 9.12	70.01 ± 6.18	62.85 ± 8.91	76.97 ± 13.08	68.28 ± 7.58

[a] Counts per gram tissue are presented as mean ± standard error. Differences from the control group were not significant by Dunnett's test.

Females

No gross lesions were observed in female pups at necropsy, but microscopic evaluation indicated exposure-related effects in the reproductive tract and mammary gland (Table 12). All females in the 1,250 ppm group showed mild to marked departure from normal morphology of the reproductive tract and/or lack of synchrony in cycle stage in the various components of the reproductive tract. The ovary of all 1,250 ppm females showed mild to moderate bilateral degeneration of the antral follicles. This lesion was not observed in the ovary of any control females. Antral follicles in 1,250 ppm females appeared to be more numerous and were in various stages of degeneration. Corpora lutea were smaller and appeared to be fewer in number than in the controls. Corpora lutea of 1,250 ppm animals were characterized by the presence of large foamy leuteinized cells with cell cords separated by well-developed capillaries and sparse connective tissue, and appeared most similar to the morphology expected for diestrus. These corpora lutea appeared to be hormonally active and not undergoing cyclic regression at the normal rate, if at all. There was little evidence of the vacuolar degeneration and apoptosis of luteal cells that usually accompany normal cyclicity. Whereas control animals showed remnants of at least one, and often two, waves of corpora lutea from previous cycles, no evidence for such remnants was seen in the animals exposed to 1,250 ppm genistein. The ovarian interstitial compartment of 1,250 ppm females was sparse and atrophic, characterized by smaller diameter, fewer cells, and a looser structure that suggested edema. Primordial, growing, and antral follicles were enumerated in five sections from each ovary of 12 to 15 animals in each exposure group, and no exposure-related differences were apparent (Table 13). Only follicles that included a normal ovum were recorded in the follicle counts; this likely accounted for the fact that follicle counts did not reflect the histopathologic observation of increased antral follicles.

The remainder of the reproductive tract in many 1,250 ppm females showed effects consistent with the disturbance of ovarian function and contrasted markedly with controls. Both uterine and vaginal structures showed an inappropriate combination of changes that reflected influences of estrus, metestrus, and diestrus. Abnormal cellular maturation in the vagina of nine 1,250 ppm animals was observed and labeled as mucocytic metaplasia, a term that denotes marked departure from normal cyclic morphologic changes in the epithelium. This included hypertrophy of the mucinous layer that reflects the progesteronal influence during proestrus. The apparent increased progesteronal drive in the 1,250 ppm group is consistent with the observation of persistent corpora lutea. In addition, four animals in the 625 ppm group were diagnosed with moderate vaginal mucocytic metaplasia in the absence of detectable ovarian degeneration. The increased mucocytic metaplasia in the 625 and 1,250 ppm groups was significant.

Genistein treatment was found to affect the mammary gland in females. Both whole mounts and hematoxylin and eosin-stained sections were evaluated and led to similar diagnoses. The most prominent effect of genistein on the female mammary gland was hyperplastic proliferation of alveolar complexes into compact lobules, with significantly increased incidences of this lesion in groups exposed to 250 ppm or greater. The incidence of hyperplasia of the epithelium of mammary gland alveoli was significantly increased in the 1,250 ppm group.

TABLE 12
Incidences of Selected Nonneoplastic Lesions in Female Rat Pups in the Reproductive Dose Range-Finding Feed Study of Genistein

	0 ppm	5 ppm	25 ppm	100 ppm	250 ppm	625 ppm	1,250 ppm
Ovary[a]	15	15	15	15	15	15	15
Degeneration, Antral Follicles, Bilateral[b,d]	0	0	0	0	0	0	15** (2.1)[c]
Vagina	15	15	15	14	15	15	14
Metaplasia, Mucocyte[d]	0	0	0	0	1 (2.0)	4** (3.0)	9** (3.0)
Mammary Gland	15	15	14	15	15	15	15
Hyperplasia, Lobules[d]	0	1 (1.0)	1 (1.0)	0	3* (1.3)	6** (1.7)	8** (2.3)
Hyperplasia, Alveoli[e]	6 (1.3)	8 (1.4)	6 (1.7)	6 (1.2)	7 (1.0)	10 (1.2)	11** (2.0)
Kidney	15	15	15	15	15	15	15
Mineralization, Renal Tubule[e]	13 (1.1)	15 (1.3)	13 (1.2)	15 (1.1)	15** (1.5)	15** (1.5)	15** (1.6)

* Significantly different (P<0.05) from the control group by Shirley's test
** P<0.01
[a] Number of animals with tissue examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked
[d] Significant exposure concentration-related trend (P<0.01) by the Jonckheere-Terpstra test
[e] P<0.001

TABLE 13
Follicle Count Summary of Female Rat Pups in the Reproductive Dose Range-Finding Feed Study of Genistein[a]

	0 ppm	5 ppm	25 ppm	100 ppm	250 ppm	625 ppm	1,250 ppm
n	15	14	14	15	12	14	15
Primordial	17.13 ± 2.23	9.00 ± 1.29	18.71 ± 2.86	18.33 ± 2.70	17.58 ± 4.17	19.50 ± 4.23	23.73 ± 3.00
Growing	2.67 ± 0.45	2.14 ± 0.52	4.36 ± 0.63	3.20 ± 0.54	4.42 ± 1.00	2.64 ± 0.59	3.53 ± 0.71
Antral	2.53 ± 0.49	3.29 ± 0.54	3.14 ± 0.71	3.67 ± 0.55	3.75 ± 0.48	2.71 ± 0.47	2.33 ± 0.51

[a] Counts are presented as mean ± standard error. Differences from the control group were not significant by Dunnett's test.

Renal tubule mineralization was observed in a high proportion of females in all groups; however, this lesion was significantly increased in severity in groups exposed to 250 ppm or greater (Table 12).

Summary of Results of Neurotoxicology, Behavioral, and Immunotoxicology Studies

The following studies were conducted in parallel with the reproductive toxicology study described above. An identical test system was used, although the behavioral and immunotoxicology studies used only 0, 25, 250, and 1,250 ppm groups. The results are reported in detail elsewhere, as indicated by the references.

Neurotoxicology Study

One male and one female from each litter in the reproductive toxicity assessment (sacrificed on PND 50) were selected for assessment of the volume of the sexually dimorphic central nucleus (SDN) of the medial preoptic area of the hypothalamus and hypothalamic content of vasopressin and β-endorphin (Appendix B and Scallet *et al.,* 2003). The results indicated that a possible effect of genistein was to reduce (i.e., feminize) the volume of the SDN in male animals receiving the intermediate doses of genistein (25, 100, and 250 ppm), while the higher doses had no effect. However, there was a lack of consensus among multiple raters of this endpoint, so that caution is necessary in interpreting this result (Appendix B). While no effects on hypothalamic β-endorphin content were detected, vasopressin levels were significantly elevated in the 1,250 ppm group. These results were consistent with both the known effects of estradiol and the findings (see below) of the behavioral assessments that indicated increased sodium preference in genistein-exposed rats.

Behavioral Study

In behavioral assessments (Flynn *et al.,* 2000), groups of 12 pregnant rats were fed soy-free diets containing 0, 25, 250, or 1,250 ppm genistein beginning on GD 7, and offspring were fed these diets through PND 63. Male and female offspring were assessed for levels of sexually dimorphic behaviors: open field activity, play behavior, running wheel activity, and consumption of saccharin- and sodium chloride-flavored solutions. Intake of the sodium-flavored solution was increased, with rats in the 1,250 ppm group drinking significantly more than those in the 0 ppm group; intake of plain water was unaffected. While there was a statistically significant dose effect on play behavior, no exposed group was significantly different from the control group in pairwise comparisons. Running wheel activity and saccharin-flavored solution consumption showed significant sex differences but no significant effects of genistein treatment. Gestational duration, total and live pups per litter, and total and live litter sex ratios were not significantly affected; however, average birth weight per live pup and offspring body weights from PNDs 42 to 77 were significantly decreased in the 1,250 ppm group. Body weight and food intake for the dams were also significantly decreased in the 1,250 ppm group.

Immunotoxicology Study

For immunotoxicity evaluations, a third set of animals was treated as described above with 0, 25, 250, and 1,250 ppm genistein in the diet. In these studies, both the dams (after exposure for 65 days) and the pups (on PND 63, after exposure for 77 days) were evaluated. Evaluations (n=10 per sex per exposure group) included spleen and thymus weights as well as assays for humoral mediated immunity (spleen antibody-forming cell response to sheep red blood cells), cell-mediated immunity (proliferative response to anti-CD3 stimulation), nonspecific immunity (natural killer cell activity), quantitation of cell subpopulations (lymphocyte subpopulations, macrophages, and natural killer cells), and hematopoietic function (bone marrow cell number and numbers of macrophage, granulocyte-monocyte, and erythrocyte progenitors).

On the day of sacrifice, whole spleens or bone marrow cells were placed in tubes with the appropriate medium, packed in wet ice and shipped to the Medical College of Virginia Campus/Virginia Commonwealth University (Richmond, VA) for assay evaluation on the following day. Exposure to genistein resulted in statistically significant increases in the relative spleen weight (% body weight) in F_1 generation males at the low and high exposure concentrations and in all exposed groups of F_1 females. The pups of both sexes, but not the dams treated only as adults, showed enhanced T-cell activity in all exposed groups as measured by anti-CD3 (a T-cell receptor) antibody-mediated spleen cell proliferation. The biological consequences of this apparent developmental effect of enhanced cell-mediated immunity are not clear; they could be beneficial or adverse (by enhancing autoimmune responses) depending on the host environment. Changes in B and T cell populations and alterations in bone marrow progenitor cell populations were also seen in the pups, but not the dams, and these effects varied according to dose and sex (NTP, 1998; Guo *et al.,* 2002, 2005). In the dams, increases in natural killer cell activity were observed at 250 and 1,250 ppm, but this effect was not observed in pups. Thus, effects on immunological endpoints were detected at exposure concentrations as low as 25 ppm genistein, although the biological consequences of these changes were not evaluated.

Serum Genistein Determination

Serum isoflavones were also determined in animals from the 0, 25, 250, and 1,250 ppm groups of the immunotoxicity study at sacrifice on PND 63 (Holder *et al.,* 1999). It was determined that 97% or more of the total circulating genistein existed as conjugates (primarily glucuronides) and that 1% to 3% was aglycone, the form of genistein that interacts with estrogen receptors. Serum concentrations ranged from approximately 10 nM in the 25 ppm group to 6 to 9 μM in the 1,250 ppm group, which spans the range of serum concentrations of humans consuming soy products.

DISCUSSION

The doses of genistein ingested by the rats in this study spanned a range that covered estimated human isoflavone exposures resulting from consumption of soy foods in adults (approximately 1 mg/kg per day) and of soy infant formula (6 to 9 mg/kg per day) (Setchell *et al.,* 1997) up to approximately 170 mg/kg per day. The mean dose ingested by dams during pregnancy was near 50% of the dose ingested by the dams during the lactation period or by the pups after weaning. While the ingested doses in the highest dose groups are well above typical human ingestion levels, the serum levels of genistein achieved over the dose range tested in the present study, even at the high end of the dose range (approximately 10 µM total genistein at 1,250 ppm in pups at termination of the study), are within a range achievable in humans consuming soy products (Setchell *et al.,* 1997). Whitten and Patisaul (2001) have also pointed out the need for higher ingested doses of genistein in rodents to produce blood levels found in humans.

Pups were also presumably exposed to genistein *in utero* and while nursing. Limited data are available on placental transfer of genistein, but these data indicate that the fetus is exposed to genistein after maternal ingestion of genistein or soy. Genistein was detected in fetal plasma and the brain 2 hours after oral dosing of pregnant rats (Doerge *et al.,* 2001). Total genistein concentrations were lower in fetal plasma than in the plasma of the dams, although the percent present as unconjugated aglycone was higher than in the dams, possibly due to the less efficient glucuronidation capacity of the fetus. In humans, transplacental transfer of genistein has been demonstrated in studies that have detected isoflavones in amniotic fluid (Adlercreutz *et al.,* 1999; Foster *et al.,* 2002). Transfer of genistein to human breast milk following consumption of soy has been reported (Franke and Custer, 1996; Franke *et al.,* 1998). In rodents, the degree of transfer of ingested genistein to milk is unclear. Fritz *et al.* (1998) reported significant levels of genistein in the milk of rats following dietary administration of genistein from conception (0.14 µM in the milk of dams consuming 250 ppm genistein that had serum levels of 0.42 µM genistein), and Weber *et al.* (2001) reported high concentrations of isoflavones in the plasma of infant rats and reasoned that there was efficient transfer of genistein from the mother's plasma to milk, although milk concentrations were not reported. On the other hand, Lewis *et al.* (2003) reported less efficient transfer to the milk of dams administered genistein by gavage, with a milk level of 0.63 µM in dams with plasma levels of 6.7 µM.

Multiple effects of genistein were observed in this study at the highest exposure concentration tested, 1,250 ppm, and the extent of these effects clearly eliminated 1,250 ppm for consideration for use in subsequent multigenerational and chronic toxicity studies (NTP, 2007, 2008c). Maternal body weight gain and feed consumption during pregnancy

were decreased. In males exposed to 1,250 ppm, effects that were considered in dose selection for the main studies included decreased body weight gain; decreased ventral prostate gland weight and secretory material; increased relative pituitary gland weight; increased incidences of inflammation of the dorsolateral prostate gland; hyperplasia of the alveoli and hypertrophy of the alveoli and ducts of the mammary gland and epididymal hypospermia; and abnormal spermatogenesis (depletion and retention of elongated spermatids). In females, effects at 1,250 ppm included decreased body weight gain; increased incidences of ovarian degeneration, vaginal metaplasia, and mammary gland hyperplasia; and increased severity of renal tubule mineralization. In addition, the dams in the 1,250 ppm group produced fewer litters than the control or other exposed groups. At 625 ppm, fewer effects were observed, with increased incidences of mammary gland hyperplasia and renal tubule mineralization as the only effects in males and increased relative pituitary gland weight and increased incidences of vaginal metaplasia, mammary gland hyperplasia, and renal tubule mineralization in females. At 250 ppm, the sole effects in the reproductive toxicity study were increased incidences of mammary gland hyperplasia/hypertrophy and renal tubule mineralization in males and females. At 25 ppm, the incidence of hypertrophy of the male mammary gland was significantly increased relative to controls. In parallel studies conducted under identical conditions, immune cell alterations (NTP, 1998; Guo *et al.,* 2002, 2005), an apparent decreased volume of the sexually dimorphic central nucleus of the medial preoptic area of the hypothalamus in males (Appendix B), and decreased thyroid peroxidase activity were reported at 25 ppm genistein, although no thyroid hormone changes or thyroid gland lesions were found to be associated with this decrease in thyroid peroxidase activity (Chang and Doerge, 2000). While the effects of genistein at 625 ppm were mild and would not have been considered to have a major impact on reproductive capacity, given the facts that effects were observed at lower exposure concentrations, that the goals of the long-term studies included the evaluation of the long-term consequences and potential magnification of subtle effects, that the limited sample size in the dose range-finding study restricted the power of the study to detect effects, and that an extended exposure period would be used in the early generations of the planned multigenerational and chronic studies, 500 ppm was selected as the high exposure concentration for the multigenerational and chronic studies (NTP, 2007, 2008c). A low exposure concentration of 5 ppm, where no significant effects were observed in the current reproductive dose range-finding study, and an intermediate exposure concentration of 100 ppm were selected.

Data from the current reproductive dose range-finding study described above, together with data on immunological, behavioral, and neurotoxicological endpoints and serum isoflavone levels generated in studies conducted under identical conditions, were the sole data considered in selecting doses for the subsequent multigenerational and chronic toxicity studies (NTP, 2007, 2008c). In recent years, particularly over the time period during which the studies described in this report were conducted, there have been multiple studies of the effects of genistein or soy extracts containing genistein on male and female reproductive organs in experimental animals. These studies have come to mixed conclusions on the degree to which genistein induces adverse effects in reproductive tissues. These

differing conclusions may in part reflect the differences in experimental design, including species and strain used, route of administration, exposure window, and base diets used in the studies. Nonetheless, it is of interest to compare the results of the current study to those reported by others using various developmental exposure models.

The ratio of litters produced to number of plug-positive dams assigned to exposure groups was significantly lower in the 1,250 ppm group. Since dosing was begun after implantation, this suggests a possible effect on post-implantation viability or resorptions. However, no effect of treatment on litter size was observed in the 1,250 ppm litters that were produced, nor were significant treatment effects on these endpoints noted in the 1,250 ppm groups of the parallel immunotoxicity and behavioral studies, nor in the study of Takagi *et al.* (2004) where a similar dosing regimen (1,250 ppm dietary genistein in a soy-free diet from GD 15 through PND 11) was used. Thus, it appears that the reduced number of litters produced in the 1,250 ppm group in the current reproductive dose range-finding study may have been a chance observation. Maternal body weight gain during pregnancy was significantly depressed in the 1,250 ppm group, and while mean pup birth weight of the 1,250 ppm group was slightly depressed, but not significantly less than that of the controls in the reproductive study, mean body weight was significantly less in the 1,250 ppm group in the behavior study (Flynn *et al.,* 2000).

No significant effect on anogenital distance (AGD) was found in the present study, although mixed and relatively modest effects of genistein or soy on AGD have been reported. The administration of dietary genistein (1,000 ppm) from GD 1 to PND 56 (Casanova *et al.,* 1999) or a soy-containing diet from GDs 0 to 20 (Weber *et al.,* 2001) increased (masculinized) anogenital distance in female pups. Casanova *et al.* (1999) also found a significantly increased anogenital distance in female pups of dams fed a soy-containing diet versus a soy-free diet. On the other hand, Levy *et al.* (1995) reported a decreased AGD in female pups born to dams that had been treated with 5 mg genistein by subcutaneous injection on GDs 16 to 20. Levy *et al.* (1995) found that 5 mg of genistein, but not 25 mg, administered to dams from GDs 16 to 20 by subcutaneous injection decreased the AGD in newborn males. On the other hand, it was reported that male pups of dams receiving a diet containing approximately 600 ppm soy phytoestrogens throughout gestation had increased ratios of anogenital distance to body weight just before birth at GD 20.5, although no significant difference was observed on PND 3 (Weber *et al.,* 2001). A diet containing 200 ppm soy phytoestrogens had no effect. The overall results suggest that phytoestrogens may be able to masculinize or hyperfeminize female pups depending on the treatment conditions, but the long-term biological meaning of these small and sometimes transient effects on anogenital distance is not clear.

In the reproductive dose range-finding study reported here, genistein had no significant effect on the onset of male puberty, as assessed by preputial separation. Likewise, while the female pups in the 1,250 ppm group showed a mean time of vaginal opening approximately 3 days earlier than controls, no exposed group was significantly different from

controls in pairwise comparisons. Wisniewski *et al.* (2003) reported a delayed time of preputial separation along with reduced testosterone concentrations and impaired reproductive behavior in Long Evans rats exposed to 5 or 300 ppm dietary genistein during gestation and lactation. Genistein effects on the timing of male puberty have generally not been observed in other studies (Casanova *et al.,* 1999; Lewis *et al.,* 2003; Masutomi *et al.,* 2003), although Masutomi *et al.* (2003) did report preputial separation accompanying a lower body weight than controls in rats fed 1,000 ppm genistein from GD 15 to PND 10. In female rats, the timing of vaginal opening, which is measured by the age or the body weight at which vaginal patency is achieved and occurs near the time of the first ovulation, is the most readily measured marker of the attainment of puberty. The timing of the onset of puberty is complex and can be altered by the levels or timing and pattern of release of controlling hormones from the hypothalamus and pituitary gland as well as direct effects on the ovaries (Goldman *et al.,* 2000). Levy *et al.* (1995) administered genistein by subcutaneous injection to pregnant dams on GDs 16 through 20 at two dose levels (5 mg and 25 mg, or about 12 and 60 mg genistein/kg body weight per day) and found a delay in vaginal opening only at the lower dose. Other studies in mice or rats with injected, gavage, or dietary genistein or with dietary soy protein isolate have generally reported an acceleration of vaginal opening or no effect (Lamartiniere *et al.,* 1998a; Casanova *et al.,* 1999; Badger *et al.,* 2001; You *et al.,* 2002a; Lewis *et al.,* 2003; Nikaido *et al.,* 2004). In two recently reported experiments using developmental exposure windows somewhat different from that used in the current study, acceleration of vaginal opening was reported within the exposure concentration range used in the current study. You *et al.* (2002a) reported accelerated time of vaginal opening in females exposed to 300 or 800 ppm genistein in feed from GD 0 through the experiment. The 300 ppm diet yielded a genistein dose of between 20 and 40 mg/kg body weight, depending on the stage of the experiment. Lewis *et al.* (2003) reported accelerated vaginal opening in pups injected subcutaneously with 2 mg/kg daily from birth to PND 7 and then gavaged with 40 mg/kg through PND 21. In the same experiment, an identical exposure regimen using 10-fold lower doses had no effect on the timing of vaginal opening. Takagi *et al.* (2004), on the other hand, did not observe an acceleration of vaginal opening when exposing rats to 1,250 ppm genistein in a soy- and alfalfa-free diet from GD 15 to PND 11, and Nagao *et al.* (2001) saw no acceleration of vaginal opening after gavage dosing of rat pups with up to 100 mg/kg genistein on PNDs 1 through 5.

The female reproductive tract showed histological changes in the 1,250 ppm group of the current reproductive dose range-finding study that indicates a significant impairment of reproductive processes at that exposure concentration. Given the preferential affinity of genistein for estrogen receptor β (Kuiper *et al.*, 1997, 1998), the relatively high concentration of this receptor in the ovary (Kuiper *et al.*, 1997), and the high level of genistein aglycone available to interact with the estrogen receptor (Doerge *et al.,* 2001), the ovary would be expected to be a likely target for genistein action. Prolonged cycles and increased time in estrus or diestrus have been observed in animals treated prepubertally with genistein (Lamartiniere *et al.,* 1995; Murrill *et al.,* 1996; You *et al.,* 2002a; Kouki *et al.,* 2003; Takagi *et al.,* 2004; and Nikaido *et al.,* 2004). Genistein-induced ovarian degeneration and abnormal cycling in rats,

as observed in the present study, have been described previously following neonatal treatment of rats with high doses of genistein (three subcutaneous doses of 5 mg/animal on PNDs 2, 4, and 6) (Lamartiniere *et al.,* 1995). Similar doses administered to older prepubertal animals and dietary administration of 25 or 250 ppm from conception through weaning did not result in ovarian toxicity, although mammary gland differentiation was affected by both treatments (Fritz *et al.,* 1998). On the other hand, Awoniyi *et al.* (1998) reported that dietary genistein at 5 ppm from GD 17 through weaning or continuing until PND 70 had lasting effects on the ovary. The effects reported, degenerating follicles and a persistent interstitial compartment, were similar to those seen in the present study. Those effects were persistent in animals that had been removed from dosed feed at weaning, suggesting that *in utero* and/or lactational exposure was responsible for the effects. Nagao *et al.* (2001) administered genistein to Sprague-Dawley rat pups at 12.5 to 100 mg/kg per day by gavage on PNDs 1 through 5 and necropsied the animals at 21 days or 18 weeks of age. In the older animals, all dosed groups showed an increase of abnormal cycles and a decreased fertility index (number of animals pregnant/number of animals copulated). Some animals in the 50 and 100 mg/kg groups showed atrophic ovaries with atretic follicles and no corpora lutea, but lesions were not observed in the lower dose groups. Lamartiniere *et al.* (1998b) detected ovarian toxicity only at high (5 mg/rat) doses of genistein administered subcutaneously to neonatal rats, and Kang *et al.* (2002) reported no ovarian lesions with maternal gavage exposures of 0.4 or 4 mg/kg body weight per day. Jefferson *et al.* (2002) treated neonatal mice (PNDs 1 through 5) with subcutaneous injections of 1, 10, or 100 µg per day and found induced expression of estrogen receptor α, an increased number of ovulated oocytes at the lowest dose, and a decrease in the number of ovulated oocytes at the higher doses. A dose-related increase in multioocyte follicles was also observed, but unlike the induction of estrogen receptor α, this effect was not observed in estrogen receptor β knockout mice. The present results are in agreement with those of Fritz *et al.* (1998) in that no ovarian toxicity was noted in doses lower than 1,250 ppm. The specific experimental factors (e.g., genetic, dietary, environmental) that account for the differences in the doses at which adverse ovarian effects were observed are not clear. Despite the lack of morphologic evidence of ovarian toxicity at 625 ppm in the current study, a significantly increased incidence of vaginal metaplasia was observed at 625 ppm. A similar low incidence of mucinification of the vaginal epithelium in the absence of ovarian toxicity in adult female rats treated orally for 28 days with 400 or 1,000 mg genistein/kg body weight per day was reported by Okazaki *et al.* (2002).

Observed effects of genistein on organ weights were relatively few in the current reproductive dose range-finding study. Consistent with an estrogenic effect (Wiklund *et al.,* 1981), relative pituitary gland weights showed an increasing linear dose trend, with significant differences from controls in the 625 ppm and 1,250 ppm groups in females and males, respectively. In males, both the relative and absolute ventral prostate gland weights showed decreasing linear trends and were significantly lower in the 1,250 ppm group. Casanova *et al.* (1999) found no significant effects of genistein on ventral prostate gland weights of pups exposed to 200 or 1,000 ppm under similar

conditions to those used in the present experiment, but they reported a marginally significant increase in ventral prostate gland weight at 200 ppm when the individual pup was used as the unit of analysis. The current study data show slight but nonsignificant elevated ventral prostate gland weight at 100 ppm and dorsolateral prostate gland weights at several intermediate exposure concentrations. In addition, quadratic dose trends were not significant for either the ventral or the dorsolateral prostate glands, and none of these results was altered when the individual pup was used as the unit of analysis. Histologically, treatment-related effects on inflammation of the dorsolateral prostate gland, a lesion present in some control animals, and depletion of secretory fluid in the ventral prostate gland were noted only at 1,250 ppm. Thus, under the exposure conditions used in the present study, genistein had relatively little effect on the prostate gland. This is generally consistent with the literature on genistein effects on the prostate gland. Lund *et al.* (2001) reported that rats exposed to a soy diet containing 600 ppm total phytoestrogens through gestation and throughout life had decreased prostate gland weight relative to rats receiving a phytoestrogen-free diet. Nagao *et al.* (2001) did not observe changes in the rat adult ventral prostate gland weight, the only prostate gland lobe examined, at oral genistein doses between 12.5 and 100 mg/kg per day on PNDs 1 to 5. Strauss *et al.* (1998) found that while ventral prostate gland weight was reduced in NMRI mice subcutaneously injected with 50 or 500 mg/kg per day on PNDs 1 to 3, hyperplasia and abnormal prostate gland histology, similar to that produced by neonatal treatment with potent estrogens, was observed only in the high dose animals as adults. Based on parallel studies with adult male mice, the authors of the latter study concluded that neonates were much less sensitive than adults to the estrogen-like actions of genistein and that adverse effects on the prostate gland in neonates did not occur at doses likely to be encountered through the diet. Kang *et al.* (2002) dosed dams by gavage with 0.4 or 4 mg genistein/kg body weight per day from GD 6 to PND 20 and examined the F_1 offspring at several ages. The prostate gland weight was significantly increased in the 4 mg/kg group at PND 70 but not on PND 100, indicating a transient effect. A similar transient effect on prostate gland weight in mice has also been reported (Kyselova *et al.,* 2004). Fritz *et al.* (2002) administered dietary genistein at 250 ppm and 1,000 ppm for 2 weeks after weaning and found a reduction in the growth of lateral prostate gland type 1 buds at the high dose but no evidence of toxicity.

Effects of genistein on the testis and the epididymis at 1,250 ppm were noted in the histologic evaluation in the reproductive dose range-finding study, consistent with a possible disruption of or delay in spermatogenesis, although this was not reflected in the testicular spermatid head count or the epididymal spermatocyte counts. Sperm are just beginning to appear in the epididymis at this age, and the dilute sperm number may contribute to considerable variability in the spermatocyte counts in the homogenates. In any case, interpretations of these changes are complicated by the peripubertal age of the animals. Degenerating germ cells are common in pubertal animals undergoing the first wave of spermatogenesis, with the cell type undergoing degeneration highly dependent on the age of the animal (Russell *et al.,* 1987). At the relatively late pubertal stage at which the animals in this study were examined, it would be expected that predominantly elongated spermatids would be degenerating (Russell *et al.,*

1987). This was observed to be the case in 2 of 15 control animals examined, but more so in the high dose animals, and degeneration of earlier generations of germ cells was also seen. Roberts *et al.* (2000) found no effect of 5 ppm dietary genistein administered from GD 17 to PND 21, PND 70, or PND 130, with all animals necropsied at PND 70 or 130, and Nagao *et al.* (2001) found no effect on sperm counts or serum testosterone after neonatal gavage administration of genistein at doses ranging from 12.5 to 100 mg/kg per day. On the other hand, Fisher *et al.* (1999) reported increased testes weight at PND 75 in animals given subcutaneous injections of 4 mg genistein/kg body weight between PNDs 2 and 12. A transient reduction in efferent duct epithelial cell height was observed in early life and was abolished by PND 25. Additionally, Atanassova *et al.* (2000) found that both a soy-containing diet and genistein administered to neonates by subcutaneous injection at 4 mg/kg per day from PNDs 2 to 18 retarded spermatogenesis in neonatal Wistar rats. Kang *et al.* (2002) found no effects on sperm number or the distribution of cell types or their numbers in adult rats whose mothers were exposed to 0.4 or 4 mg genistein/kg body weight per day by gavage from GD 6 to parturition and then from PNDs 2 to 20. Robertson *et al.* (2002) found that soy-containing feed could partially reverse the deficient spermatogenesis observed in aromatase knockout mice. A recent report indicated that rats treated with a mixture of soy-derived isoflavones (45% genistein, 200 and 2,000 ppm in the diet) during adulthood (treatment for a year starting at 10 weeks of age) resulted in no effects on testicular or epididymal weights or on sperm count, motility, or morphology (Faqi *et al.,* 2004). Likewise, feeding genistein at 250 or 1,000 ppm from PNDs 21 to 35 had no effect on testis weight or histology, in contrast to the decreased weight and altered morphology induced by 75 ppb DES (Fritz *et al.,* 2003). Kyselova *et al.* (2004) found in a multigeneration drinking water study that 2.5 and 25 mg genistein/kg body weight reduced the weights of testes, prostate glands, and seminal vesicles in 30-day-old male CD-1 pups, but the effect was not seen at 90 days of age in adults. Decreased acrosome staining was reported in sperm from these mice, but there was no dose-response for this effect, and there was no impact on fertility. Adachi *et al.* (2004) treated ICR mice subcutaneously with 1 mg genistein or 50 µg DES per day on PNDs 1 to 5 and showed that both compounds had similar effects on gene expression in the testes, including downregulation of androgen receptors and estrogen receptor α. However, while DES showed histologic effects and induced an increase in apoptotic cells in the testis, genistein was without effect. Fielden *et al.* (2003) found no effects of lower gavage doses of genistein, ranging from 0.1 to 10 mg/kg, administered during gestation and lactation on gene expression or spermatogenesis in mice. Finally, in a primate study by Sharpe *et al.* (2002), male marmosets fed soy formula had a depressed neonatal testosterone surge and increased Leydig cell number in the testis. In a follow-up study of these animals, no effects of soy formula feeding on the timing or progression of puberty, fertility, or development or length of the penis were observed (Tan *et al.,* 2006). Testis weight and Sertoli and Leydig cell numbers were increased in the marmosets exposed to soy formula as infants. Thus, the data available to this point show mixed evidence for a biological effect of soy and genistein on the rodent and primate testis, but with the exception of the study of Wisniewski *et al.* (2003) mentioned previously, which reported adverse reproductive effects in male Long-Evans rats at 5 ppm genistein, clear adverse reproductive outcomes or other long-term testicular toxicity have not been demonstrated.

Mineralization of renal tubules, or nephrocalcinosis, is a sex-related lesion common in untreated female rats and influenced by diet composition (Ritskes-Hoitinga and Beynen, 1992). Dietary genistein at 250 ppm and above induced a significant increase in the incidence and/or severity of this lesion in both sexes. Control males did not show signs of nephrocalcinosis. This lesion has been reported to be induced by estrogen treatment in males (Ritskes-Hoitinga and Beynen, 1992) and could be related to the estrogenic activity of genistein. On the other hand, treatment-related increases in incidences of nephrocalcinosis in males have not been noted after dietary administration of 17β-estradiol or 17α-ethinyl estradiol to rats (Schardein, 1980; Biegel *et al.,* 1998). While the nephrocalcinosis induced in males by genistein was mild and would not be expected to impact longevity or fertility, the fact that this lesion has been observed in animals treated under the same protocol with nonylphenol (Latendresse *et al.,* 2001) is of interest.

The mammary gland showed treatment-related effects in both sexes. The effects of genistein on the female mammary gland have been reported in studies evaluating the ability of the isoflavone to act as a chemopreventive agent against chemically induced mammary carcinogenesis in rodents. The basic findings have been that, like more potent estrogens, genistein administered perinatally and prepubertally inhibits mammary carcinogenesis induced by subsequent carcinogen exposure (Brown and Lamartiniere, 1995; Brown *et al.*, 1998; Fritz *et al.*, 1998; Lamartiniere *et al.*, 1998a). This effect presumably is related to the stimulation of mammary gland differentiation, perhaps related to modulation of growth factor response pathways (Brown *et al.*, 1998). The effects of dietary genistein on mammary carcinogenesis and development were previously observed at dietary concentrations of 25 and 250 ppm, doses at which no ovarian or uterine effects were seen (Fritz *et al.,* 1998). Hilakivi-Clarke *et al.* (1999a) have also shown that prepubertal genistein inhibits later carcinogen-induced mammary carcinogenesis and stimulates mammary gland differentiation in the rat. However, this group has also reported that restricting genistein exposure to the prenatal period results in a less differentiated mammary gland in mice (Hilakivi-Clarke *et al.,* 1998) and rats (Hilakivi-Clarke *et al.,* 1999b) and an increased susceptibility to carcinogen exposure in rats at PND 50 (Hilakivi-Clarke *et al.,* 1999b). In the present study, mammary gland structures were not quantified, but the qualitative assessment of the mammary gland was in agreement with the results reported by Fritz *et al.* (1998) for a similar dietary exposure regimen, in that more differentiated lobules were more prevalent at PND 50. In this study, the number and size of terminal end buds were also increased and significant effects were seen only at 625 and 1,250 ppm in females. The observed lobulo-alveolar development of the mammary gland is generally what would be expected from increased exposure to progesterone (Shyamala, 1999), so that the mammary gland effects observed here, particularly in the 1,250 ppm group, could be, in part, secondary effects resulting from genistein's effect on the ovaries or from increased production of prolactin.

Treatment-related effects on duct and alveolar mammary gland epithelium of male rats in the present study were seen at 25 ppm or greater. While the incidence and severity of hypertrophy diagnosed in the male mammary gland were statistically significant at 25 ppm, the incidences of hyperplasia were only statistically significant at 250 ppm or greater. Since the biological significance of hypertrophy in the absence of hyperplasia is unclear, the clearly biologically significant effects of genistein thus occurred at 250 ppm. You *et al.* (2002b) have also reported that the mammary gland of male rat pups was more sensitive than the mammary gland of females to genistein. Male pups exposed to 800 ppm, but not those exposed to 300 ppm dietary genistein *in utero* and during lactation from GD 1 through PND 22, showed increased ductal branching when examined at the end of the exposure period. The literature on normal development and xenobiotic or estrogenic effects on male mammary tissue is sparse. In studies of dietary 17β-estradiol (10 and 50 ppm) (Biegel *et al.,* 1998) and/or 17α-ethinyl estradiol (0.08 ppm) (Schardein, 1980), examination of the mammary glands in adult males indicated feminization. Cardy (1991) reported that treatment of male rats with a dopamine antagonist resulted in male mammary glands with a tubuloalveolar structure typical of females and speculated that an increase in prolactin resulting from the drug treatment may have been responsible for the feminizing effect. Cardy (1991) also suggested that the male mammary gland may be a valuable marker tissue for endocrine active compounds. Genistein has been reported to increase prolactin concentrations in ovariectomized female rats fed 750 ppm genistein (Santell *et al.,* 1997) and to stimulate prolactin production in pituitary gland cells in culture (Stahl *et al.,* 1998), but the hormonal status of the animals in the present study was not evaluated.

In summary, the results of this dose range-finding study provide evidence of the potential of dietary genistein to affect multiple estrogen-sensitive organs in both male and female rats. Results reported in the literature leave questions as to the nature of adverse effects that may be produced by genistein and the doses at which such effects occur. The study reported here indicated effects in both males and females, and these effects will be further evaluated in multigenerational and chronic studies (NTP, 2007, 2008c) conducted in the same experimental model as this dose range-finding study. The 1,250 ppm exposure concentration was clearly ruled out for further testing based on the effects on body weights, histopathologic observations in males and females, and a reduction in the proportion of mated dams producing litters. While the effects observed at 625 ppm would not be predicted to significantly impair reproduction, the observation of significant effects at 250 ppm (hyperplasia in the mammary gland of both sexes), together with the suggestion of subtle effects at this exposure concentration and lower in the parallel immunotoxicity and neuroanatomical studies, suggested that a high exposure concentration between 250 and 625 ppm would be appropriate for the purposes of the multigenerational reproductive toxicology and chronic studies. Accordingly, the highest exposure concentration for the multigenerational reproductive toxicology and chronic studies was set at 500 ppm. A low exposure concentration of 5 ppm, where no significant effects were observed in the reproductive dose range-finding study, and an intermediate exposure concentration of 100 ppm were also selected.

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APPENDIX A
CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION OF GENISTEIN

Genistein for this study was obtained from Toronto Research Chemicals, Inc. (North York, Ontario, Canada), in one lot (1-BP-118-3). Identity and purity analyses were conducted by the study laboratory, the National Center for Toxicological Research (Jefferson, AR). Reports on analyses performed in support of the reproductive dose range-finding study of genistein are on file at the National Center for Toxicological Research.

Lot 1-BP-118-3 of the chemical, a pale-yellow solid, was identified as genistein by the study laboratory using mass spectrometry and ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy. All spectra were consistent with the structure of genistein, and the mass spectrum was similar to that of a reference standard sample of genistein obtained from Sigma Chemical Company (St. Louis, MO). The mass, ^{1}H-, and ^{13}C-NMR spectra are presented in Figures A1, A2, and A3, respectively.

The purity of lot 1-BP-118-3 was determined by the study laboratory using ^{1}H- and ^{13}C-NMR spectroscopy and high-performance liquid chromatography (HPLC). HPLC was performed with a Phenomenex ODS-3 column (250 mm × 4.6 mm, 5-µm particle size, Phenomenex, Torrance, CA) and a solvent system of 70% water/30% acetonitrile adjusted to pH 3.0 using 0.1% formic acid. The flow rate was 0.2 mL/minute; ultraviolet detection at 260 nm was used.

^{1}H- and ^{13}C-NMR spectroscopy results both indicated the presence of a single impurity (1.1%, mole/mole) containing an ethyl group that was tentatively identified as ethanol. The purity profile obtained using HPLC indicated a purity of at least 99%. The overall purity of lot 1-BP-118-3 was determined to be 99% or greater.

To ensure stability, the bulk chemical was stored at –70° C, protected from light, in opaque white plastic bottles.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared approximately every 1 to 3 weeks by mixing genistein with soy- and alfalfa-free irradiated Purina 5K96 feed (Table A1). A premix was prepared by hand and then blended with additional feed in a Patterson-Kelly twin-shell blender for 45 minutes using an intensifier bar. The 250 and 1,250 ppm dose formulations were prepared directly, and the 5, 25, 100, and 625 ppm dose formulations were prepared by geometric dilution of the more concentrated formulations. Formulations were stored at 4° ± 2° C, protected from light in stainless steel cans for up to 22 days.

Homogeneity and stability studies of the 5 ppm dose formulation were performed by the study laboratory using HPLC as previously described. Stability studies were conducted for 17 days under simulated animal room conditions (21.1° to 24.4° C), 8 weeks at 10° to 21° C, and 32 weeks at 4° C. Homogeneity was confirmed, and stability was confirmed for at least 22 days for dose formulations stored in stainless steel cans protected from light at 4° ± 2° C.

During the reproductive dose range-finding study, the dose formulations were analyzed five times by the study laboratory using HPLC. All 13 dose formulations analyzed were within 10% of the target concentrations (Table A2).

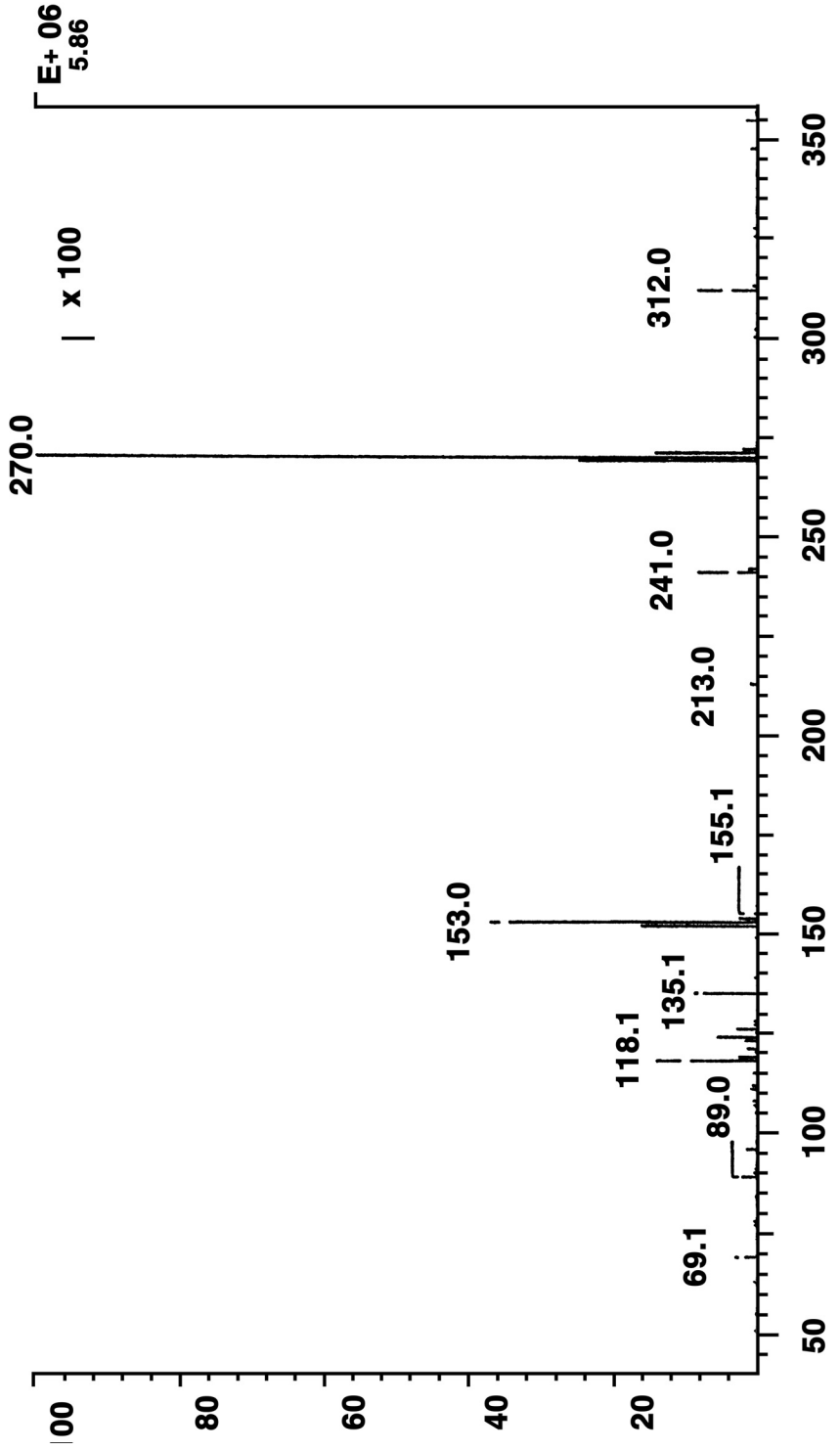

FIGURE A1
Mass Spectrum of Genistein

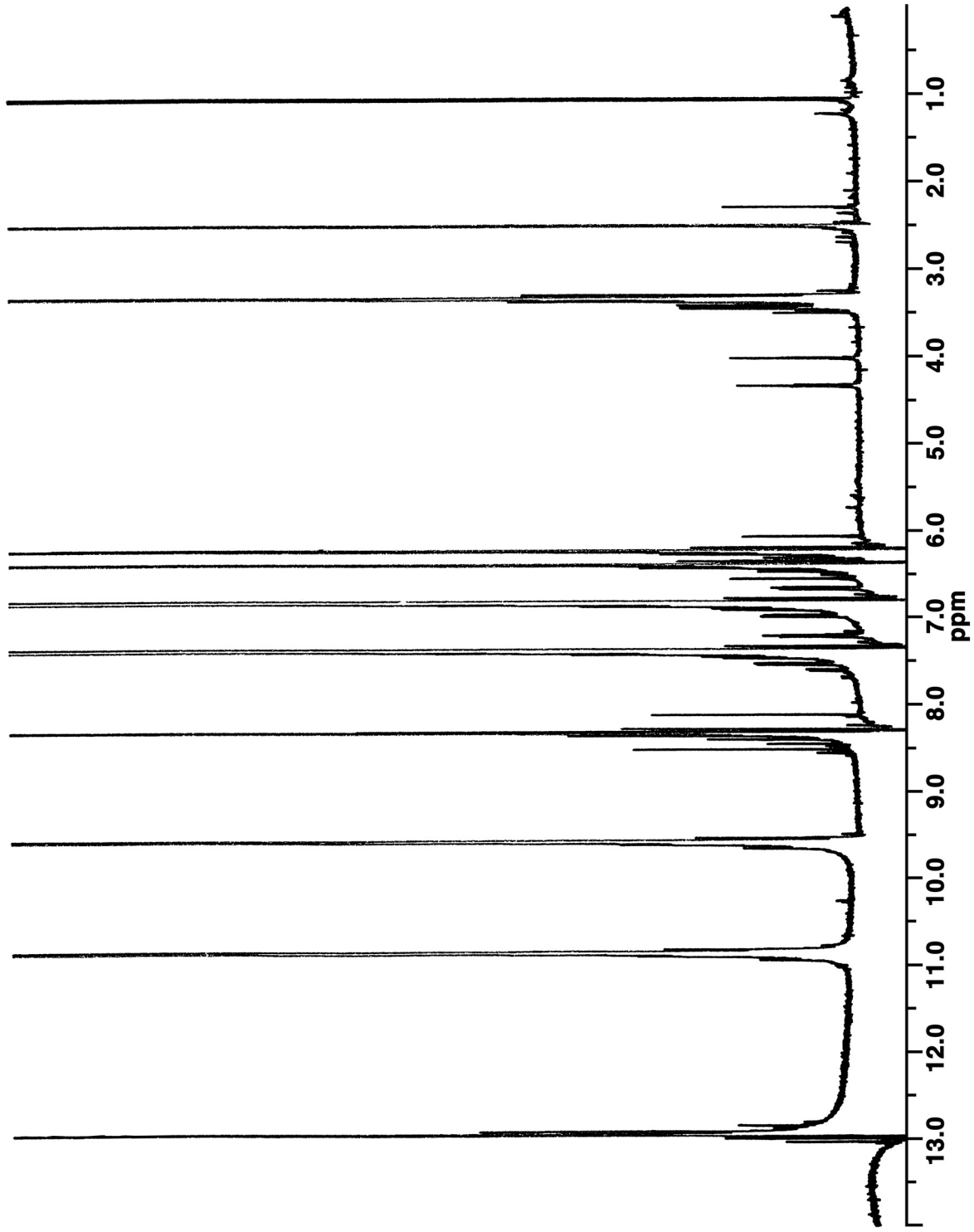

FIGURE A2
^{1}H-Nuclear Magnetic Resonance Spectrum of Genistein

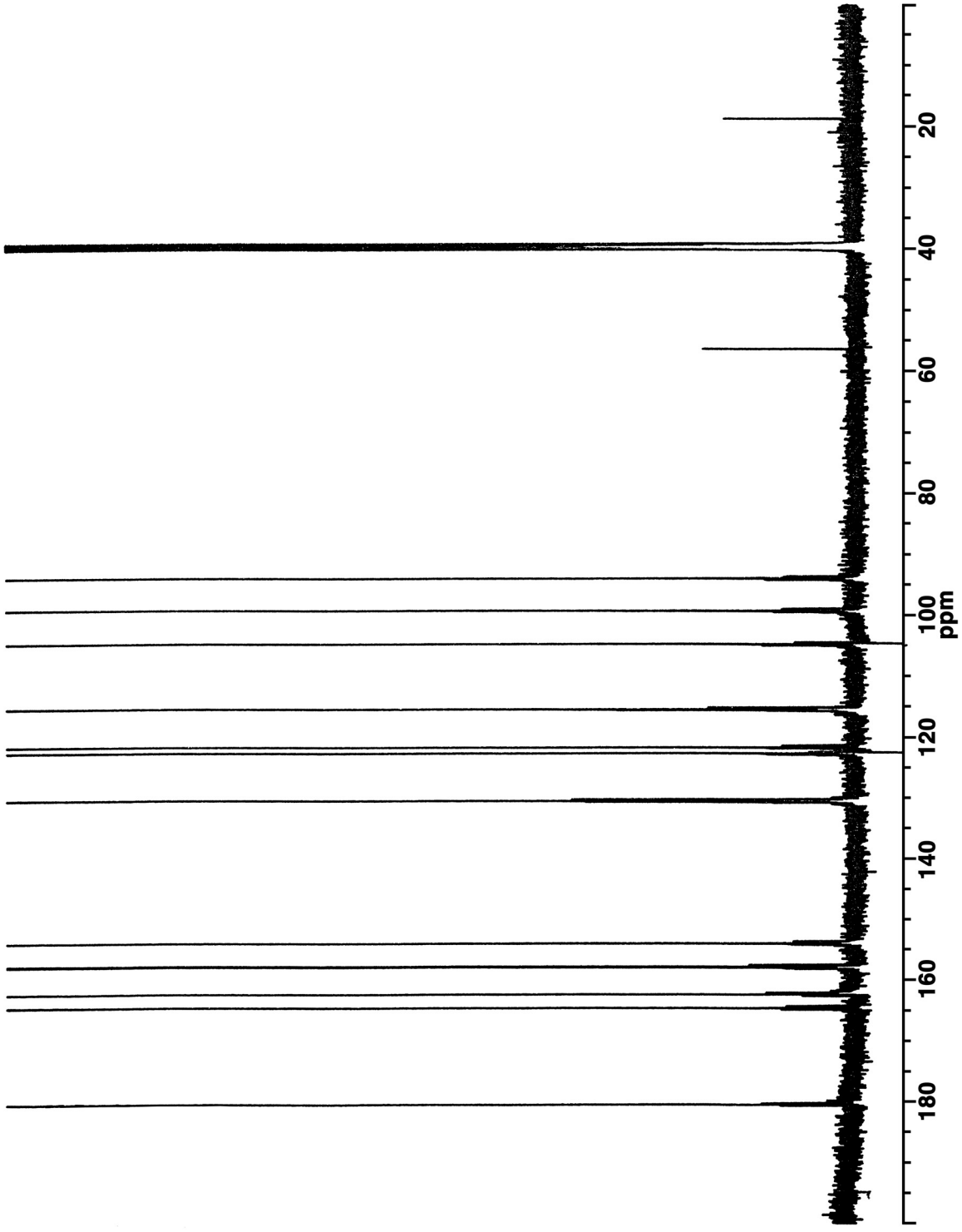

FIGURE A3
^{13}C-Nuclear Magnetic Resonance Spectrum of Genistein

TABLE A1
Preparation and Storage of Dose Formulations in the Reproductive Dose Range-Finding Feed Study of Genistein

Preparation
Genistein was mixed with soy- and alfalfa-free irradiated Purina 5K96 feed to give the required concentrations. A premix of feed and genistein was prepared, then layered into the remaining feed and blended in a Patterson-Kelly twin-shell blender with the intensifier bar on for 45 minutes. The 250 and 1,250 ppm dose formulations were prepared by mixing the appropriate quantity of bulk genistein into the Purina 5K96 feed. By geometric dilution, the 625 ppm dose formulation was derived from the 1,250 ppm dose formulation, and the 5, 25, and 100 ppm dose formulations were derived from the 250 ppm dose formulation. The dose formulations were prepared four times during the study.

Chemical Lot Number
1-BP-118-3

Maximum Storage Time
22 days

Storage Conditions
Stored at 4° ± 2° C, protected from light, in stainless steel cans

Study Laboratory
National Center for Toxicological Research (Jefferson, AR)

TABLE A2
Results of Analyses of Dose Formulations Administered to Rats in the Reproductive Dose Range-Finding Feed Study of Genistein

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
June 17, 1997	June 17, 1997	250	255	+2
		250	255	+2
	June 20, 1997	5	5.09	+2
		25	24.8	–1
		100	102	+2
June 23, 1997	June 23, 1997	625	618	–1
		1,250	1,263	+1
July 15, 1997	July 15, 1997	25	22.9	–8
		250	243	–3
		1,250	1,244	0
August 6, 1997	August 12, 1997	5	4.74	–5
		100	92.7	–7
		625	613	–2

[a] Results of duplicate analyses.

APPENDIX B
NEUROHISTOLOGICAL AND NEUROCHEMICAL TOXICITY OF GENISTEIN

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NEUROHISTOLOGICAL AND NEUROCHEMICAL TOXICITY OF GENISTEIN

OVERVIEW

Genistein is a plant-derived estrogenic isoflavone commonly found in consumer products such as soy milk, dietary supplements for treating menopausal symptoms, etc. In choosing neurotoxicological endpoints to evaluate potentially adverse effects of genistein, it is logical to first examine endpoints that have been well-established for detecting effects associated with estrogen, viewed as the prototypical parent compound for the endocrine disruptors such as genistein.

Estrogen has been widely documented to influence the early development of the sexually dimorphic central nucleus (SDN) of the medial preoptic area (MPOC) in rats; exposure during the critical perinatal period results in a masculine adult SDN (larger than in a female) and masculine adult reproductive behaviors.

Vasopressin is a neurosecretory peptide synthesized in neurons of the paraventricular nucleus of the hypothalamus and secreted into the bloodstream from axons extending into the posterior lobe of the pituitary gland. Estradiol has also been widely observed to influence the synthesis or content of vasopressin found in the hypothalamus of developing or adult rats, resulting in functional alterations of fluid retention and blood pressure.

The endogenous opiate peptide β-endorphin is synthesized both in neurons of the hypothalamic arcuate nucleus, as well as in pituitary cells, primarily of the neurointermediate lobe. It has been reported that exposure to estradiol valerate selectively damaged hypothalamic β-endorphin-containing neurons, resulting in reduced hypothalamic content of β-endorphin, increased μ-opiate receptors, and increased sensitivity to the analgesic effects of morphine injections. Lower doses of estradiol have been found to *increase* hypothalamic β-endorphin.

Our neurotoxicological approach to the evaluation of genistein effects on rats of the range-finding study has thus focused mainly on the neurohistological endpoint of the volume of the SDN and the neurochemical endpoints of hypothalamic content of vasopressin and β-endorphin.

The results indicate that a possible effect of the range-finding dietary exposure to genistein was to reduce the volume of the adult SDN in male animals receiving the intermediate doses of genistein. However, an examination of the methodological issues involved in the measurement of the SDN revealed a lack of consensus between multiple raters. Thus we suggest caution at present in accepting uncritically the finding that genistein reduced male SDN volume, except in the heuristic sense that further research on the potential effects of genistein on SDN development should be undertaken in the hopes of resolving the differences between raters.

While there were no effects of dietary genistein on hypothalamic β-endorphin content, vasopressin levels were significantly elevated in the 1,250 ppm genistein group ($P<0.05$). This data is consistent with the known effects of estradiol and accompanied findings by the NCTR neurobehavioral toxicology group that estrogenic “endocrine disruptors” such as genistein increased sodium preference in exposed rats.

METHOD OF EVALUATION OF THE SEXUALLY DIMORPHIC CENTRAL NUCLEUS OF THE MEDIAL PREOPTIC AREA

In order to assess the effects of dose, duration of exposure, etc., on the MPOC, we first required a practical method of quantifying the changes in cytoarchitectonic structures. A desirable method should provide reproducible, accurate, and unbiased measurements suitable for statistical analysis, with sufficient ease and rapidity so that a

large number of brains could be processed. Most previous approaches to obtain an estimate of MPOC volume (Table B1) have utilized an extension of a two-dimensional procedure. The MPOC is outlined on each of several individual sections; then the area of MPOC on each section is multiplied by the section thickness. The total volume is then determined as the sum of the volumes of each individual section. However, Table B1 reveals considerable intra- and inter-laboratory variation in the published values for the volume of the MPOC, the most commonly studied sexually dimorphic hypothalamic nucleus, using this method.

There are also a number of studies which have used computer programs to three-dimensionally reconstruct various brain regions from serially cut tissue (Toga and Arnicar-Sulze, 1987; Villa *et al.*, 1987; Bertossi *et al.*, 1989; Toga *et al.*, 1995; Yamaguchi and Goto, 1997). These approaches offer the advantage of placing measurements within a three-dimensional framework, where information obtained from all the sections evaluated is included. Although the feasibility of such an approach to evaluate the rat MPOC has been demonstrated (Robinson *et al.*, 1986), the widespread application of such techniques was precluded until recently by the requirement for custom software and extensive computing resources. Here, we will briefly describe a protocol for three-dimensional imaging, reconstruction, and measurement of the anteroventral hypothalamus and its component nuclei.

Tissue Preparation

Brains were sagitally cut through the midline and 3 mm lateral to the midline. The resulting slabs of tissue were placed in tissue cassettes and transferred to a Citadel 1000 tissue processor (Shandon, Inc.). The tissue was then dehydrated through 70%, 95%, and 100% ethyl alcohol, cleared in xylene and infiltrated with paraplast maintained at 57° to 62° C under vacuum. Processed tissue was then embedded with Paraplast X-TRA in a Histocenter 2 tissue embedding center (Shandon, Inc.).

Twenty µm thick serial sections were cut using a RM 2145 rotary microtome (Leica). Damaged sections (≈ 1/100 slides) were recorded using blank slides. Sections were stained with 0.04% Cresyl Violet containing acetic acid (pH to 3.4) on a DRS-601 automatic stainer (Sakura Finetek, Inc.).

Prior to any three-dimensional analysis, the computer and microscope were calibrated with a standard traceable to the National Bureau of Standards (Geller Microanaytical Laboratory, Peabody, MA). Following calibration, 4× digital photomicrographs were taken of the stained sections containing the MPOC using a VANOX-T microscope (Olympus Optical Co.) attached to a DXC-970MD 3CCD color video camera (Sony Corp.) and a CMA-D2 camera adapter (Sony Corp.).

The software used for digitizing and three-dimensional rendering of the sections was MCID-M5+ (4.0 Beta 2.4) for Windows NT (Imaging Research, Inc.). We manually aligned each digitized image with the image that immediately preceded it. Reference points included the third ventricle and the paraventricular nucleus. Freehand tracings using a computer mouse were made around the third ventricle (3V) and the MPOC. The alignment of these images was further refined by a second alignment using fiducial points placed on the drawings in the center of the MPOC and the bottom center of the third ventricle.

Using the computer, the sections were then stacked 20 µm apart in three-dimensional space. A computer-generated three-dimensional rendering of the MPOC was created from the stack of sequential sections. The volume of the MPOC was determined based on the 4× system calibrations. Volume measurements of the MPOC are reported as the number of voxels (times their size in cubic mm) that were contained within the surface rendered over the MPOC outlines within the stack of sequential sections. Accuracy was confirmed by successful measurement of the volume of a cube with known dimensions. The sizes of outlines of the female rat MPOC, as traced independently by two separate experimenters following the procedures described above, were significantly correlated (Pearson $r=0.79$, $n=17$, $P<0.01$).

Table B1

MPOC Volume (mm^3 x 10 ± S.E.M.)								
Male	Female	N	Animal Age	Fixative	Tissue processing	Section Thickness	Histological Stain	**Authors**
4.4±0.25	--	25m	*PND 89-13*	10% formalin *perfusion*	*Paraffin*	*50 µm*	*Thionin*	*Anderson, 1986*
≈5.0	--	6m	*PND 6*	*10% formalin immersion*	*Paraffin*	*5 µm*	*Hematoxylin And eosin*	*Vancutsem, 1997*
4.55±0.38 -and- 5.70±0.86	0.46±0.06 -and- 0.89±0.11	11m 11f	7-8 months	10% formalin perfusion	Frozen sectioned	60 µm	Cresyl violet	Jarzab, 1990
6.12±2.01	**0.40±0.03**	**4m 4f**	***PND 24***	***10% formalin perfusion***	***Paraffin***	***20 µm***	***Cresyl violet***	***Present Study***
6.46±1.66	**0.56±0.15**	**4m 4f**	***PND 50***	***10% formalin perfusion***	***Paraffin***	***20 µm***	***Cresyl violet***	***Present Study***
8.3±1.7	3.2±1.0	4m 4f	--	*10% formalin perfusion*	*Paraffin*	10 µm	Cresyl violet	Gorski, 1980
--	≈4.0	--	PND 49	10% formalin immersion	frozen sectioned	20 µm	Cresyl violet	Faber, 1993
≈10.00	≈4.80	10m 6f	PND 49	10% formalin immersion	frozen sectioned	20 µm	Cresyl violet	Faber, 1991
≈16.00	≈5.80	3m 4f	PND 75-105	10% formalin perfusion	frozen sectioned	60 µm	Thionin	Jacobson, 1981
≈18.00	≈6.00	5m 6f	PND 60	10% formalin perfusion	frozen sectioned	60 µm	Thionin	Dohler, 1982
19.3±1.9	3.8±2.0	5m 5f	3.5-5.5 months	10% acrolein perfusion	Celloidin	20 µm	Cresyl violet	Robinson, 1986
≈21.00	≈10.50	12m 5f	PND 14-31	10% formalin perfusion	frozen sectioned	60 µm	Thionin	Ahmed, 1991
21.1±1.4	3.9±0.5	6m 6f	Adult	10% formalin perfusion	frozen sectioned	60 µm	Thionin	Bloch, 1988
≈22.00	≈12.00	16m 16f	PND 200	10% formalin perfusion	frozen sectioned	60 µm	Thionin	Davis, 1995
≈24.00	≈6.00	5m 6f	Adult	10% formalin perfusion	frozen sectioned	60 µm	Thionin	Jacobson, 1980
24.32	6.51	12m 12f	PND 30	10% formalin perfusion	frozen sectioned	60 µm	Thionin	Rhees, 1990a
24.38	3.43	--	PND 15	10% formalin perfusion	frozen sectioned	60 µm	Thionin	Rhees, 1990b
25.30±3.14 -and- ≈35.0	NA -and- ≈5.0	21m	12 weeks	10% formalin perfusion	frozen sectioned	60 µm	Thionin	Tarttelin, 1988
25.50	12.33	6m 6f	PND 10	10% formalin perfusion	gelatin embedded	60 µm	Thionin	Maecker, 1993
≈32.00 -and- ≈18.0	≈15.00 -and- ≈6.0	5m 6f	2 months	10% formalin perfusion	frozen sectioned	60 µm	Thionin	Dohler, 1984

In the present study, plus four additional independent sets (n=5 males, 5 females for each set) of control groups, mean male MPOC volumes ranged from 5.95 to 8.83 $mm^3 \times 10^{-3}$ while mean female MPOC volumes ranged from 0.29 to 0.71 $mm^3 \times 10^{-3}$.

The volumes of the MPOC in male and female rats reported here fall within the range of MPOC volumes as measured by other investigators (Table B1). In adult rats, the range of MPOC volumes as measured by other investigators extended from 4.55×10^{-3} mm^3 to 32×10^{-3} mm^3 in males and 0.46 to 15×10^{-3} mm^3 in females (Table B1).

The rather broad distribution of MPOC volumes reported in the literature suggests it may be important to consider various factors that may contribute to intra- and interlaboratory variation. Some error between measurements may occur during the original drawing of the areas. For instance, deciding where to outline the nuclei requires subjective determinations based on detecting shifts in cell density, staining characteristics, and cell morphology of the particular nucleus. To make the measurement procedures as objective as possible, one might have two experimenters draw the structures and then create a composite drawing based on common features (Gorski *et al.*, 1978). However, to permanently require two investigators would substantially slow the evaluation process. We have instead tried to document that multiple raters could achieve suitable inter-rater reliability of measurement.

Effects of Dietary Genistein Exposure on the SDN

Pregnant dams were maintained on dosed chow from gestational day 7 (GD 7) until the pups were weaned on postnatal day (PND) 24. Pups were then maintained on the chow until sacrifice at PND 50. To eliminate any influence from isoflavones that may occur in commercially prepared NIH-31 rat chow, which is the standard rodent diet used at NCTR, a separate isoflavone-free chow was developed. This modified chow (Purina 5K96; Test Diets, Purina Mills, Richmond, IN) had all soy and alfalfa protein removed and replaced with casein and soy oil replaced by corn oil. A total of 70 offspring (35 males and 35 females) were exposed via the 5K96 chow to seven different doses of genistein. The doses were 0, 5, 25, 100, 250, 625, and 1,250 ppm. All animals were overdosed with 0.6 mL sodium pentobarbital (60 mg/ml, Veterinary Laboratories, Inc.). They were then perfused transcardially with 40 mL of 0.9% saline followed by 350 mL of 10% buffered formalin at a flow rate of 40 mL/minute using a Masterflex Digi-staltic pump (Cole-Parmer Instrument Co.). The tissue was then prepared for three-dimensional reconstruction and volume measurement as described above.

Statistics

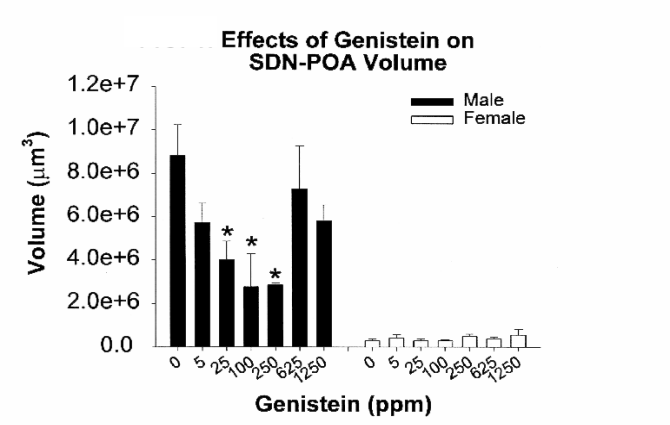
SigmaStat 2.03 (SPSS, Inc.) was used to perform all statistical tests. Two-way analyses of variance were carried out for MPOC volume data. Student-Newman-Keuls *post hoc* tests were carried out on all significant comparisons. Results were considered significant at $P<0.05$.

Results

The MPOC volumes of female rats exposed to chronic dietary genistein did not vary across the different doses (Figure B1). However, there were clear dose-related effects of genistein on the development of the male MPOC. MPOC volumes were clearly smaller in male rats exposed to 25, 100, or 250 ppm of genistein in their diet as compared to the control rats.

At the higher doses of 625 and 1,250 ppm, male MPOC volumes did not differ from their controls. There was a clear sexual dimorphism in the MPOC between male and female controls (8.83 ± 1.40 versus $0.29 \pm 0.08 \times 10^{-3}$ mm^3). This significant sexual dimorphism disappeared at the 100 and 250 ppm doses of genistein.

Figure B1



Effects of Dietary Genistein Exposure on β-endorphin and Vasopressin Levels in the Rat Hypothalamus

A previous neurobehavioral toxicology study demonstrated that dietary exposure of male or female rats to genistein increased their intake of a sodium chloride solution in preference to water (Flynn *et al.*, 2000). For genistein, only the highest exposure group (1,250 ppm) was significantly affected, although there was a trend for the lower dose groups to prefer sodium chloride as well. Previous research has indicated that acute exposure to 17-β-estradiol stimulates the synthesis of vasopressin in hypothalamic paraventicular and supraoptic magnocellular neurons. This brain-derived vasopressin is then transported to the posterior pituitary gland, which secretes the peptide into the circulation (Hashimoto *et al.*, 1981; Roy *et al.*, 1999). The circulating vasopressin can then cause increases in a number of important physiological parameters such as blood pressure, sodium retention, fluid intake, etc. (Silverman *et al.*, 1990). Because these adverse effects of estradiol exposure are only temporary (during the period immediately after exposure) and they are reversible (with suspension of dosing), they are known as "activational" effects.

There is also some evidence for more permanent alterations in hypothalamic peptides produced by estrogenic compounds (Brawer *et al.*, 1993). For example, exposure to a single 2 mg/kg intramuscular (IM) injection of estradiol valerate resulted in a loss of 60% of β-endorphin immunopositive neurons in the arcuate nucleus of the hypothalamus when measured 8 weeks later: an "organizational" effect of estrogens (Desjardins *et al.*, 1993). The estradiol valerate procedure also resulted in polycystic ovaries, with reduced binding of gonadotropins to cystic thecal cells (Convery and Brawer, 1991).

Because vasopressin and β-endorphin have been useful biomarkers of both certain activational as well as organizational effects of estrogenic compounds, we hypothesized that they might be responsive to the effects of exposure to the phytoestrogen genistein, as well. Reasoning that altered functioning of the vasopressin system may have been responsible for the increased salt intake after exposure to the estrogenic "endocrine disrupters," we sought to determine if dietary exposure to genistein would indeed increase hypothalamic vasopressin content, as estradiol had been shown to do. Moreover, would the dose-response relationship by which genistein increased hypothalamic vasopressin content be similar to the dose-response by which genistein increased sodium intake? Because β-endorphin content had also been a useful biomarker for revealing neuronal loss in the hypothalamus following exposure to estradiol valerate (Desjardins *et al.*, 1993), we measured it as well.

Materials and Methods

Animals and Groups

Female adult Sprague Dawley rats were date-mated in the NCTR breeding colony. Experimental dietary exposure to genistein (0, 25, 250, or 1,250 ppm) began 5 days after the dams were observed to be plug-positive. The diets were prepared in isoflavone-free diet as described above. Offspring were culled to 4 males and 4 females per litter, and the dams remained on the experimental chows through weaning, whereupon the offspring were continued on the same chow until they were sacrificed at 77 days of age. The vivarium was maintained under controlled environmental conditions (temperature 22° C, relative humidity 50%, 12-hour light/dark cycle with lights out at 1800 hours); rats were housed in plexiglass cages with hardwood chips for bedding. The experimental rat chows were available *ad libitum*.

Dissection, Preparation, and Analysis of Brain Tissue

Rats were sacrificed by asphyxiation with carbon dioxide, and their brains were rapidly removed. They were immediately frozen on dry ice and stored at –70° C. The entire hypothalamus was later dissected out (Glowinski and Iversen, 1966) and placed in a microfuge tube containing 1.0 mL of cold 0.1N HCl. Following sonication for 5 seconds each, the samples were centrifuged at 12,000 g for 15 minutes at 5° C in a refrigerated microcentrifuge. The supernatatants were divided into 100 µL aliquots and stored at –70° C until assayed in duplicate by ELISA for β-endorphin and arginine-8-vasopressin, using kits from Peninsula Laboratories (San Carlos, CA). The brains from 28 rats were used for neuropeptide studies: controls (n=4), 25 ppm (n=8), 250 ppm (n=8), and 1,250 ppm (n=8). These groups each contained equal numbers of males and females.

Statistical Analysis

The β-endorphin and vasopressin data were evaluated by analysis of variance using Sigmastat Software (SPSS Science, Chicago IL). There were no differences between the sexes in hypothalamic levels of either peptide, so the data is presented only as a function of the amount of genistein exposure (one-way ANOVA with four levels of dose). *Post hoc* comparisons were performed according to Fisher's Least Significant Difference (LSD) approach using a 0.05 level of significance.

Results

β-Endorphin

Levels of hypothalamic β-endorphin were not significantly altered by the amount of genistein in the diet ($F(3,24) = 1.774$, $Pp>0.10$). Figure B2 illustrates the mean levels (in ng/g wet weight of tissue) and the standard errors of the means for each of the four experimental groups.

Vasopressin

On the other hand, hypothalamic vasopressin levels were significantly different between the four groups of rats fed with different amounts of genistein in their diet ($F(3,24) = 3.122$, $P<0.05$). Figure B3 illustrates the mean levels (in ng/gram wet weight of tissue), as well as the standard errors of the means for each of the four dietary groups. Pairwise comparisons between the groups using Fisher's LSD approach indicate that the bulk of the effect can be attributed to the elevated level in the high-dose (1,250 ppm) genistein group. The 1,250 ppm group was significantly elevated from the control and 250 ppm groups ($P<0.05$; Figure B3) but not from the 25 ppm.

Discussion

β-Endorphin

The levels of hypothalamic β-endorphin (approximately 200 to 400 ng/g) we measured in the present study are similar to previous results obtained from rats and mice using radioimmunoassay methods (Scallet, 1982; Hong *et al.*, 1985; Alessi *et al.*, 1988; Holson *et al.*, 1988; Desjardins *et al.*, 1992; Schipper *et al.*, 1994; Caputo *et al.*, 1996). Previous research has reported that estrogenic compounds either increased or decreased hypothalamic

Figure B2

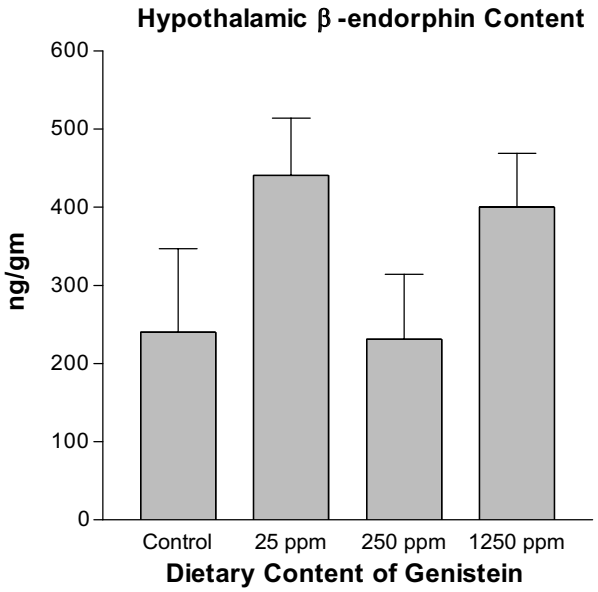
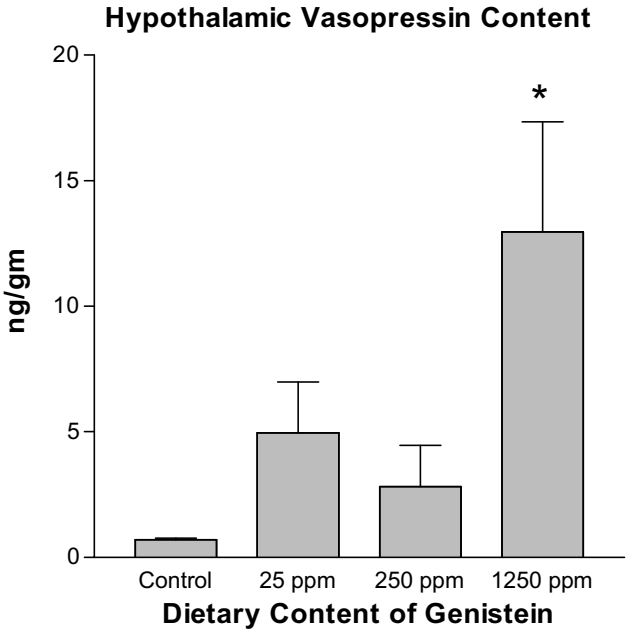


Figure B3



β-endorphin levels, depending on the circumstances. Thus, a single dose of 10 µg of estradiol benzoate administered intramuscularly. to ovariectomized/adrenalectomized rats elevated both β-endorphin and α-melanocyte stimulating hormone (α-MSH) in the hypothalamus. The melanophore-darkening hormone α-MSH, like β-endorphin, is a peptide product of the proopiomelanocortin or POMC gene, found both in the hypothalamus and the pituitary gland. This estradiol benzoate regimen also induced sexual receptivity in a subgroup of the treated animals; the increases in β-endorphin and α-MSH were the largest in the arcuate, ventromedial, and preoptic hypothalamic regions of the sexually receptive females (Medina *et al.*, 1998).

In contrast, a single intramuscular injection of 2 mg of estradiol valerate (Brawer *et al.*, 1993; Schipper *et al.*, 1994) resulted in neuronal loss (primarily of β-endorphin immunopositive cells) in the arcuate nucleus of the hypothalamus. The neuronal loss was detectable by radioimmunoasssay as a decrease in hypothalamic content of β-endorphin immunoreactivity (Desjardins *et al.*, 1993).

The studies reporting neuronal loss and decreased β-endorphin immunoreactivity used a 200-fold higher dose of estradiol than the study that showed an estradiol-induced increase of β-endorphin immunoreactivity. The former "organizational" effect was a result of the pathological loss of peptide neurons, while the latter "activational" effect is probably occurring at a more physiological dose level of estrogen. It was uncertain whether to expect any effects of genistein on hypothalamic β-endorphin immunoreactivity under the conditions of the present study, since we were using a less potent estrogen added chronically to the diet. In any event, the normal β-endorphin levels we observed in this study suggest the absence of any pathological loss of β-endorphin neurons. Moreover, our data provide no evidence of any genistein-induced "activational" increases of POMC peptides that regulate reproductive systems in the hypothalamus.

Vasopressin

The levels of hypothalamic vasopressin we observed by ELISA were somewhat lower than in previous reports using radioimmunoassay methods (Epstein *et al.*, 1983; van der Sluis *et al.*, 1986). We are uncertain whether variation between rat strains, sample storage conditions, etc. may have accounted for the differences; however, all our samples were handled identically and as a single batch throughout the analyses. In many previous reports, estrogenic compounds have been reported to alter the levels of hypothalamic vasopressin, as well as other magnocellular neuropeptides of the hypothalamus. Thus, vasopressin is not the only neurosecretory peptide affected by exposure to estrogenic compounds. For example, some hypothalamic magnocellular neurons express and secrete primarily oxytocin instead of vasopressin. Physiologically, oxytocin mediates such things as milk elaboration and let-down reflexes in the breast, as well as uterine contractions. Using single cell RT-PCR (Glasgow *et al.*, 1999) showed that neurons primarily expressing oxytocin mRNA, also expressed at least some vasopressin mRNA, and *vice versa*. Analyses of these neurons for coexisting peptide mRNAs also revealed corticotropin releasing hormone, cholecystokinin, galanin, dynorphin, and the calcium-binding protein calbindin, as well as high voltage-activated calcium channel subunit genes.

Using immunohistochemical staining methods, Levin and Sawchenko (1993) had observed the same set of neuropeptides described by Glasgow *et al.* (1999) plus an additional blood-pressure regulating peptide, angiotensin II. Their observations suggested that corticotropin releasing hormone, cholecystokinin, and vasopressin all showed evidence of enhanced expression after treatment with 17-β-estradiol. Using *in situ* hybridization in the 17-β-estradiol-treated rhesus monkey hypothalamus, Roy *et al.* (1999) showed corticotropin releasing hormone message was increased in the magnocellular neurons, but there was no change in vasopressin mRNA amounts. Such an observation of unchanged mRNA concentration may nevertheless be consistent with the general findings reported above of increased vasopressin peptide, since the amount of vasopressin mRNA may not be rate-limiting for vasopressin synthesis.

Estradiol is not the only estrogenic compound that has been shown to alter hypothalamic magnocellular peptides. In the study of Schriefer (1991), diethylstilbesterol treatment (70 µg/day subcutaneously for 2 days) increased oxytocin but decreased met-enkephalin in magnocellular neurons, as measured by radioimmunoassay. Vasopressin was also elevated by about 10%, but the effect was not statistically reliable. Term pregnant rats, as with diethylstilbesterol treatment, had elevated oxytocin and decreased met-enkephalin levels, but in addition, they

showed statistically significant increases in both vasopressin and dynorphin levels. This group also reported mRNA measurements consistent with their radioimmunoassay findings. These findings suggested the possibility of different, but overlapping, actions of diethylstilbesterol compared to the effects of endogenous steroids elevated by pregnancy. Thus, estrogenic compounds are clearly important factors regulating hypothalamic neurosecretion, which in turn may influence neuroreproductive function.

There is very limited information regarding the effectiveness of different estrogenic compounds given at different doses by different routes of administration on hypothalamic neuropeptides. Our data indicate that chronic dietary exposure to 1,250 ppm of genistein is sufficient to increase the amount of hypothalamic vasopressin that is detectable by ELISA. Moreover, the dose-response function we report here for vasopressin elevation is identical to our earlier observation of the dose-response relationship for dietary genistein to increase rodents' preferences for drinking salt-water over tap water (Flynn *et al.*, 2000).

SUMMARY

SDN volume in rats has been a very intriguing endpoint, both when applied for understanding the role of estrogens and testosterone in normal development and in studies involving experimental exposure to xenobiotic compounds. Very often, the SDN volume endpoint has been utilized in conjunction with separate functional evaluations of such things as reproductive status or secondary sexual characteristics. Our study reported here provided some support for the notion of a biphasic dose-response effect of genistein causing a reduction of SDN volume ("feminization") of the male rat in the dose range of 25 to 250 ppm. However, enthusiasm for this effect should be tempered by the knowledge that the littermates of these same rats were not impaired in reproductive performance or in testis weights. Moreover, studies of the reproducibility of SDN volume measurement, both historic and our own more recent one, reveal poor concordance between different investigators. We believe that the present investigation raises some concern about the possibility that genistein may feminize SDN volume in male rats, but we strongly urge caution in relying on SDN volume measurement alone until corroboration from other biomarkers may be obtained.

The ELISA methodology for neuropeptide analysis, in conjunction with functional/behavioral measures, identified an effect of genistein on vasopressin that may relate to physiological/functional effects on salt balance, fluid retention, and blood pressure. These measures should continue to be useful tools for better characterizing the neurotoxicology of estrogenic endocrine disruptor compounds.

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VALIDATION STUDY OF SDN MEASUREMENT PROCEDURES

A number of groups, including our own, have investigated the potential effects of developmental exposure to estrogenic endocrine disrupters on the average size of the SDN. As we began these studies, we noticed that the SDN, not even recognized as a distinct nucleus by classical neuroanatomists, presented a considerable challenge to the morphometrist. When comparing within and between laboratories (Meredith *et al.*, 2001; Table 1), we found that there were up to 30-fold differences in the volumes that were reported. These differences were sometimes variations between laboratories, but often they were based on reports of a given lab that were simply published in different years.

To our knowledge, no systematic study of the source of this variability has been attempted. The purpose of this validation protocol is to determine the reliability of measurement of the SDN volume by partitioning the variance of the estimation of the true mean of SDN volume into separate components.

According to Winer, 1971, p. 289 (Winer, B.J., *Statistical Principles in Experimental Design*, 2nd ed., New York: McGraw-Hill, 1971), the measurement of a parameter such as the volume of the SDN nucleus can be divided into several components. The measurement equals its "true" value, plus an error component due to the measurement process, and an "anchor point" due to the main effect of the measuring instrument itself. In our case, the "anchor point" is the "judge" or decision maker of where to draw the line around the SDN. This analysis allows one to address possible systematic differences between observers in whether they typically tend to draw the nuclei in a more inclusive or more exclusive way.

In fact, when several judges have measured the same parameter in multiple subjects, Winer presents an analysis of variance to estimate the reliability of the mean of k measurements. This analysis allows us to consider the variation between groups of nuclei separately from the variability due to the contribution of multiple judges and the contribution of variability when a given measure is performed repeatedly.

In order to understand the reliability of our procedures, the same test set of slides consisting of the SDNs from 15 males was measured by each of four investigators. Analysis of Variance and correlational analyses were performed to estimate the mean volume of control as well as nonylphenol and genistein-treated male SDNs. The sections to be measured were taken from the genistein and nonylphenol rangefinding experiments (E 2122.15 and E2125.15) and coded by Dr. Delclos to ensure that all judges were blind to the treatment groups. The specific sections were obtained as follows in Table 1.

The resulting data from the remeasured SDNs indicated that the inter-rater correlation coefficients were quite low (see Figure 1).

By comparison, the earlier correlation coefficient between repeated results of two investigators using our same methodology was much higher (about 0.79, see above). However, among other differences, the earlier study only compared the ability of the two investigators each to measure a set of outlined areas on 17 slides obtained from a single female animal. The present study adopted the more stringent standard of requiring blind measurement of 15 completely separate male animals, each SDN being completely reconstructed from a variable number of slides per animal chosen independently by each investigator.

Analysis of variance of the data was performed in order to evaluate the effects of Raters as well as effects of estrogenic treatment and a possible interaction (i.e. did the raters differ in their evaluation of the presence and degree of an estrogenic effect?). There was no effect of estrogenic treatment ($F(1,10) = 2.03$, N.S.), but there was a significant effect of rater ($F(3,30) = 5.94$, $P<0.01$). No interaction was present ($F(3,30) = 1.48$, N.S.). These results can best be appreciated by looking at Figure 2.

Figure 2 reveals that while all the Raters were relatively close to one another in their estimates of SDN volume in untreated control animals (within about a twofold range from lowest to highest), their estimates of shifts in SDN volume in animals treated with estrogenic compounds were different. Raters 2, 3, and 4 failed to observe the shift in SDN volume originally reported by Rater 1. The significant effect of raters probably stems from the low scores measured by R3 in both treated and untreated rats, as well as perhaps the low scores from Rater 1 in treated rats. The absence of a significant interaction, though, demonstrates the lack of a significant difference between the raters in how the size of the SDNs was rated, with or without estrogenic treatment.

TABLE 1

Study	Dose Group (ppm)	ID #	Original Measure (X 10^6 μm^3)
2122	0	126a	10.5
2122	0	130a	6.05
2122	0	297a	9.95
2122	100	104a	4.29
2122	100	321a	1.23
2122	250	170a	3.03
2122	250	217	2.77
2122	250	225	2.75
2125	0	95a	8.99
2125	0	206	7.36
2125	0	290a	5.43
2125	200	99	0.598
2125	200	101a	1.37
2125	200	103	1.59
2125	200	297	1.34

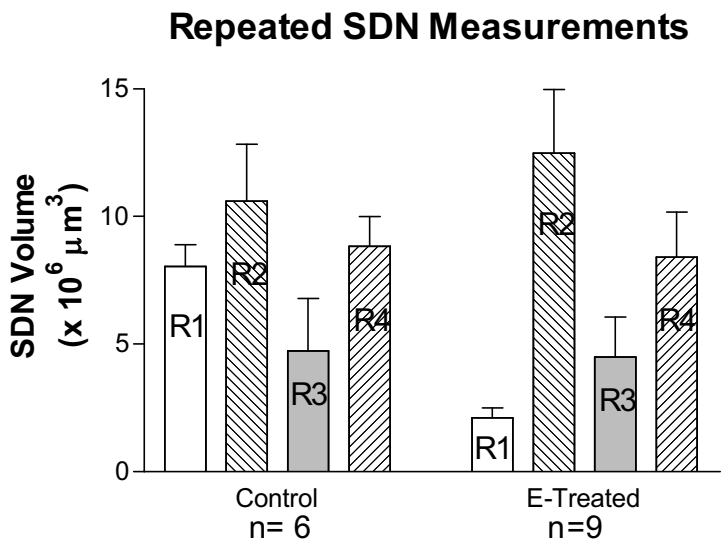
FIGURE 1

Correlation coefficients (Pearson r) between the four different raters:

	1	**2**	**3**	**4**
1	1.00			
2	-0.18	1.00		
3	0.02	0.13	1.00	
4	-0.21	-0.01	0.11	1.00

Our results failed to provide additional support for an effect of genistein on SDN volume, since additional raters did not agree well with the original scoring. While disappointing, perhaps these results are not surprising given the large variability characteristic of SDN measurement as presented in Table 1. It is hoped that refinements in the techniques may be instituted that will allow more repeatable measurements of SDN volume. In the meantime, it seems best to urge caution in the interpretation of the results of SDN volume determinations, such as those presented in the Body of this report. While shifts in SDN volume may raise concerns, supplementary functional information on such things as reproductive success and behavioral competence should be present also in order to gather more complete information.

FIGURE 2



APPENDIX C
ASSOCIATED PUBLICATIONS

List of Publications/Technical Reports derived from or including data from NCTR Experiment E-2122.01 (Range-Finding Study for the Evaluation of the Toxicity of Genistein Administered in the Feed to CD (Sprague-Dawley) Rats and associated experiments E-2122.13 (Behavioral Toxicology), E-2122.14 (Immunotoxicology), and E-2122.15 (Neurotoxicology):

Chang, H.C., and Doerge, D.R. (2000). Dietary genistein inactivates rat thyroid peroxidase *in vivo* without an apparent hypothyroid effect. *Toxicol. Appl. Pharmacol.* **168**, 244-252.

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